# A COMPARATIVE STUDY OF THE EMPTYING OF THE GALL BLADDER IN THE OPOSSUM AND THE CAT, TOGETHER WITH NOTES ON THE ANATOMY OF THE BILIARY TRACT OF THE OPOSSUM

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FIVE TEXT FIGURES AND THREE PLATES (SIX FIGURES)

In view of the clinical importance attached to the discharge of bile in man, it has seemed desirable to accumulate as much data as possible concerning this mechanism in vertebrates. Accordingly, the authors have endeavored to secure information about the evacuation of the gall bladder in the opossum—the most primitive mammal available for laboratory observation—and to study, more accurately than hitherto, certain details in the emptying of the gall bladder of the cat—that vertebrate in which complete evacuation of the viscus after a meal of yolk and cream was first demonstrated.[1]

## MATERIAL AND METHODS

The observations recorded are based upon twenty-four x-ray series (each consisting of from twenty-five to seventy-five cholecystograms). Twelve such series were made of each species. After each animal had fasted for twelve to twenty-four hours it was laparotomized and the bladder bile was replaced by halogenated oil. 'Lipiodol,' as originally employed by Whitaker ('26), was used in twenty cases and

[1] The authors are indebted to Prof. E. A. Boyden for proposing this problem and for numerous helpful suggestions.



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'light brominol' in the remaining four cases.[2] Twelve to fifteen hours later, during which interval no food was given, two x-ray pictures were made to determine the gall-bladder shadow and to serve as controls. As rapidly as possible thereafter egg yolk and cream were administered by gavage (three to four yolks and 30 to 50 cc. of cream). One-half to four minutes afterward, the third picture was taken, following which successive pictures were made at two- to three-minute intervals for the first hour, approximately every five minutes for the second hour, every ten minutes for the third hour, and every fifteen minutes for the fourth hour postcibum. In cases which required a longer period, subsequent roentenograms were made at fifteen- to thirty-minute intervals. Certain features thus observed have, in turn, led to anatomic and physiologic studies designed to explain functional differences noted in the two species.

## SEQUENCE OF EVENTS AFTER YOLK AND CREAM

*A. Cats.* In the twelve animals subjected to the above procedure the halogenated content was either completely expelled (five cases) or else only a small drop remained after contraction ceased (seven cases). But when the animals were arranged in the order of emptying time (table 1, a) it was noted that they fell into two distinct categories. In the first group (fig. 1) approximately two-thirds of the oil was expelled within forty-five minutes after it first entered the cystic duct. This compares favorably with the rate of evacuation of bile in man (72 per cent at forty-five minutes postcibum, table 3,

[2] 'Light brominol,' supplied to us by the courtesy of Drs. George M. Higgins and Fred Thompson, of the Mayo Foundation, was the roentgen-opaque substance employed by Doctor Thompson in his study of the emptying of the gall bladder in rabbits (Master's thesis, '32, University of Minnesota). This product (now made available by the Abbott Laboratories) contains 33 per cent brominized esters of olive oil and has a very low viscosity. Conversely, lipiodol is heavy and viscid. Both heavy and light oils were employed in an attempt to correlate the motility of the gall bladder with the viscosity of contained fluid. As indicated in table 1, the density of the contents did not seem to affect the rate of evacuation in either the cat or the opossum.

Boyden, '28). Similarly, the period required for complete evacuation (44 to 281 minutes) approximates that in man (one-quarter to four and one-half hours).

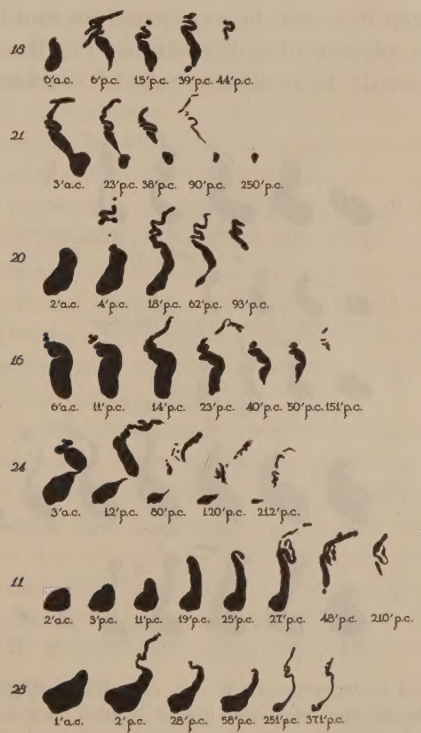


Fig. 1 Selected cholecystograms of seven cats, illustrating rapid discharge of opaque oil from gall bladder following meal of yolk and cream (arranged as in table 1, a). *a.c.*, minutes antecibum; *p.c.*, minutes postcibum. × ⅓.

In the second group, however (fig. 2), the discharge was much slower and more or less evenly distributed over a long period of time, the principal period ranging from 140 to 507 minutes (table 1, a). During this period the gall bladder was not under contraction continuously, although it has been stated that a single injection of cholecystokinin maintains

tonus in the gall bladder of the dog for over two hours; for in most cases there is evidence of intermittent contraction during these long periods. Nor do the records justify the inference that the longer periods of discharge in the second group of seven cats can be explained as due to longer intervals between phases of contraction. On the contrary, they are due primarily to a slower rate of discharge. In five of

Fig. 2 Selected cholecystograms of five cats, illustrating discharge of opaque oil from gall bladder in a slow and graded manner over a long period of time following meal of yolk and cream (arranged as in table 1, a). × ⅛.

the cases the principal period of discharge was either preceded (nos. 18 and 15) by a preliminary, or followed (nos. 16, 24, 28) by a secondary period of activity which, however, was not very effective. Incidentally, the method employed did not permit exact measurement of the intervals of contraction, but the evidence from the first x-rays—taken at two- to three-minute intervals—suggests that phases of contraction in the cat, as in the dog (McMaster and Elman, '26) are shorter than in man.

Again, limitations of method precluded an analysis of the length of time that the gall bladder of the cat remains empty, but the data at hand are consistent on the whole with Boyden's original observations ('25) that the gall bladders of cats sacrificed at varying intervals after a meal of yolk and cream were found empty between one and three-quarters to six and three-quarters hours postcibum. If anything, the new data would tend to decrease the estimate of the earliest time in which it is possible to empty the gall bladder of the cat.

Regarding the latent period of contraction, it may be stated that while the time may simulate that of man (one to two minutes), i.e., the response to the presence of food may result in almost immediate tonus changes or initial spurts of oil, generally there is an average delay of twenty-nine minutes (ranging from two to ninety minutes) before the first effective phase of contraction gets under way. This is in marked contrast to man and the monkey (Boyden, '32).

On the other hand, the length of time required for oil to traverse the cystic and common duct in cats (approximately two minutes) is much less than the time it takes in man for cystic bile to reach the duodenum after the first phase of contraction gets under way (a minimum of eight minutes postcibum; Boyden and Birch, '30). This quicker passage in the cat may be due to the absence of heisterian valves in the cystic duct or to a different regulatory mechanism at the end of the common duct.

Frequently it was noted that the gall bladder was under tonus before the animal was fed; and in one instance (no. 21, table 1, a and fig. 1) it was spontaneously discharging. Similar observations have been recorded by Boyden ('28) in man, and Birch and Boyden ('30) in cats. In the latter animal such tonus changes were found to be correlated with gastric peristalsis and could be reproduced in the gall bladder, experimentally, by stimulating the stomach of an unanaesthetized cat with a faradic current.

Lastly, no correlation was found between the length of time required to evacuate the gall bladder and its initial size, as

Table 1

## Discharge of Opaque Oil from Gall Bladder Following Meal of Egg Yolk and Cream

*a. In cats**

| An. no. | Meal | Latent period of contraction | Oil enters: Cystic duct | Oil enters: Duodenum | Duration of principal period of discharge | Total emptying time |
|---|---|---|---|---|---|---|
| **Bulk of contents emptied in short period (see fig. 1)** | | | | | | |
| 18 | 3 yolks 40 c.c. cream | Less than 4 | Less than 4 | 6 | 5 (39 - 44 p.c.) | 44 |
| 21 | 3 yolks 40 c.c. cream | Discharging when fed | | | 90 (0 - 90 p.c.) | 90 |
| 20 | 2.5 yolks 35 c.c. cream | Less than 4 | 4 | 6 | 119 (4 - 123 p.c.) | 123 |
| 16 | 2 yolks 30 c.c. cream | 13 | 13 | 15 | 30 (13 - 43 p.c.) | 151 |
| 24 | 3 yolks 40 c.c. cream | | Later than 9 | 12 | 70 (10 - 80 p.c.) | 212 |
| 11 | 2 yolks 30 c.c. cream | Less than 3 | 25 | 27 | 185 (25 - 210 p.c.) | 255 |
| 28† | 3 yolks 40 c.c. cream | Less than 2 | Less than 2 | 3 | 26 (2 - 28 p.c.) | 281 |
| *Average of 7 cats* | | | | | *75* | *165* |
| **Slow and graded discharge over long period (see fig. 2)** | | | | | | |
| 27† | 3 yolks 35 c.c. cream | | 27 | 30 | 295 (27 - 322 p.c.) | 322+ |
| 9 | 2.5 yolks 25 c.c. cream | 36 | 70 | 90 | 374 (90 - 464 p.c.) | 464 |
| 14 | 2 yolks 30 c.c. cream | | | 62 | 140 (62 - 202 p.c.) | 512+ |
| 15 | 1 yolk 22 c.c. cream | Less than 6 | | 8 | 280 (30 - 310 p.c.) | 519 |
| 17 | 3 yolks 30 c.c. cream | 8 | 14 | 16 | 507 (14 - 521 p.c.) | 521 |
| *Average of 12 cats* | | | | | *177* | *208* |

*b. In opossums***

| An. no. | Meal | Latent period of contraction | Oil enters: Cystic duct | Oil enters: Duodenum | Duration of principal period of discharge |
|---|---|---|---|---|---|
| **Early reaction (see fig. 3)** | | | | | |
| 27† | 3 yolks 40 c.c. cream | 3 | 16 | 17 | 35 (16 - 51 p.c.) |
| 16 | 3 yolks 40 c.c. cream | 6 | 16 | 18 | 45 (16 - 61 p.c.) |
| 4 | 3.5 yolks 40 c.c. cream | 10 | | 30 | 49 (30 - 79 p.c.) |
| 3 | 3 yolks 40 c.c. cream | 8 | 17 | 19 | 52 (17 - 69 p.c.) |
| 7 | 4 yolks 40 c.c. cream | 8 | 17 | Less than 23 | 56 (17 - 73 p.c.) |
| 11 | 4 yolks 40 c.c. cream | 6 | 11 | 13 | 100 (11 - 111 p.c.) |
| 17 | 3 yolks 40 c.c. cream | 9 | 22 | 24 | 99 (22 - 121 p.c.) |
| 13 | 3 yolks 40 c.c. cream | 16 | 31 | 41 | 132 (31 - 163 p.c.) |
| 12 | 3 yolks 40 c.c. cream | 7 | 11 | 13 | 188 (11 - 199 p.c.) |
| *Average of 9 opossums* | | *8* | *18* | | *84 (19 - 103 p.c.)* |
| **Delayed reaction (see fig. 3)** | | | | | |
| 5 | 4 yolks 45 c.c. cream | 21 | | 66 | 46 (61 - 111 p.c.) |
| 8 | 4 yolks 50 c.c. cream | 22 | 154 | | 196 (154-350 p.c.) |
| 28† | 3 yolks 40 c.c. cream | 12 | 21 | 23‡ | 211 (77 - 288 p.c.) |
| *Average of 12 opossums* | | | | | *101* |

All figures indicate minutes except those in first two columns

* Arranged according to total emptying time

** Arranged according to length of principal period of discharge

† Brominol in gall bladder

‡ Momentary spurt

p.c., post cibum.

may be gathered from arranging the gall bladders in group 1 according to their volumes, the volume being recorded as the amount of halogenated oil required to just fill the viscus without overdistention. For example, the bile reservoir of cat no. 18 had a volume of 1 cc. and emptied in forty-four minutes, while that of cat no. 11, with a volume essentially the same (0.9 cc.), required a period of 255 minutes for complete evacuation. Whether a much larger series would have shown a correlation between volume of the vesicle and time of emptying is a matter of conjecture.

*B. Opossums.* In marked contrast to the preceding studies are the results obtained by feeding egg yolk and cream to twelve opossums. In no case did the gall bladder react by more than partial evacuation—usually by discharging less than one-third its content of oil. Furthermore, the sequence of events would seem to predicate the existence of a different physiological mechanism than that which pertains in the cat.

In place of pronounced and rapid alterations in shape characteristic of the cat the somewhat spherical gall bladder of the opossum (fig. 3) slowly assumes a pyriform contour, requiring in nine specimens an average of twenty-six minutes (eight of which represent the latent period of contraction) before it discharges its contents into the cystic duct. Then after an average period of emptying of only eighty-four minutes, during which time only a little oil is expelled (compare last column, table 1, b, and fig. 3), it slowly reverts to its original form.

## DISCUSSION

This marked dissimilarity of reaction of the two species naturally leads to a scrutiny of the factors which might be responsible for these differences. Foremost among these is the shape and size of the gall bladder. Although the average cat and opossum have essentially the same weight (1.5 to 2.5 kg.) the gall bladder of the latter is relatively larger than the former. Of the twelve vesicles studied in each species, those of cats ranged in volume from 0.9 to 2 cc. (an average of 1.37)

while those of opossums ranged from 0.8 to 5 cc. (an average of 2.45 cc.). So that if the gall bladder of the opossum emptied only half of its contents (which is more than it usually does) it would be expelling nearly as much oil as the gall bladder of a cat which evacuated all its contents. At the least, then, the disparity in efficiency of the two vesicles is not as great as would appear from superficial observation.

A second factor is undoubtedly the difference in caliber and attachment of the cystic duct (figs. 4 and 5). In the cat the

Fig. 3 Selected cholecystograms of twelve opossums, illustrating discharge of opaque oil from gall bladder following meal of yolk and cream (arranged as in table 1, b). *a.c.*, minutes antecibum; *p.c.*, minutes postcibum. × ½.

gall bladder is elongated (usually narrower than in fig. 5) and its broad neck tapers gradually into a large-bore, convoluted cystic duct, which, in contrast to the human cystic duct is nearly as large in diameter as its own common duct. In the opossum, however, the somewhat spherical vesicle which constitutes the gall bladder, passes abruptly, with scarcely any cervical differentiation, into a straight, tenuous, cystic duct, the greatest diameter of which is usually less than one-half that of a cat of comparable size. This diminu-

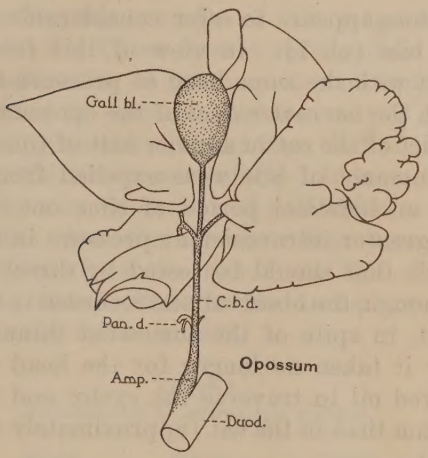


Fig. 4 Sketch of liver and extrahepatic biliary passages in the opossum. Note the straight tenuous cystic duct, the long confluent portion of pancreatic and common bile ducts, and the ampulla of Vater lying wholly without but contiguous to the duodenal wall. (See DuBois and Hunt, '32, for description of peristalsis of this portion of common duct.) *Amp.*, ampulla of Vater; *C.b.d.*, common bile duct; *Duod.*, duodenum; *Pan.d.*, pancreatic duct, dividing into dorsal and ventral divisions (note high position on common duct and the tendency toward proliferation of accessory pancreatic lobes at this point, suggesting the condition found in ruminants).

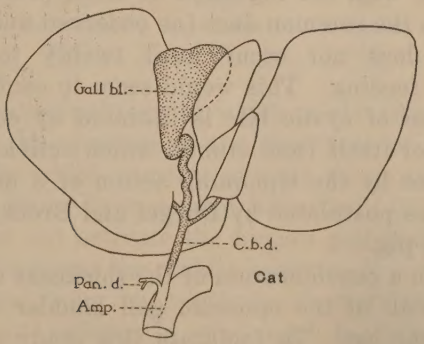


Fig. 5 Sketch of liver and extra hepatic biliary passages in the cat. Note pyriform gall bladder (in this case somewhat bilobed) which tapers in its cervical region to join the broad convoluted cystic duct.

tive outlet thus appears to offer considerable obstruction to the flow of bile (pl. 1). In view of this fact, it might be inferred that with the same head of pressure less bile would pass through the narrower duct of the opossum than through the broad duct of the cat in a given unit of time. Conversely, if an equal amount of bile were expelled from the two gall bladders in an identical period of time one would have to predicate a greater intravesicular pressure in the opossum—an hypothesis that should be tested by direct measurement. Curiously enough, the observations recorded in table 1, b, seem to show that, in spite of the somewhat thinner wall of the gall bladder it taken no longer for the head of the column of halogenated oil to traverse the cystic and common ducts in the opossum than in the cat (approximately two minutes in each species).

A possible explanation for this rapid flow of bile in the opossum is peristalsis of the common duct—a phenomenon recently described by the authors in connection with an ampullary mechanism (at the end of the common duct) that lies wholly outside of the duodenum (DuBois and Hunt, '32). This may accelerate the flow of cystic bile, but does not induce it, for although peristalsis of the common duct begins almost simultaneously with the ingestion of food, entrance of dark green bile into the common duct (as observed under nembutal anaesthesia) does not occur until twenty to twenty-five minutes after feeding. This would seem to confirm the view that evacuation of cystic bile is initiated by contraction of the gall bladder itself (and stimuli which activate its musculature) and not by the siphoning action of a muscular duct and ampulla, as postulated by Burget and Brocklehurst ('28) for the guinea-pig.

This leads to a consideration of the thickness of the muscle layer in the wall of the opossum gall bladder as compared with that of the cat. To facilitate this study specimens of physiologically distended and collapsed gall bladders of comparable size were selected and subjected to identical histological techniques. Measurements of the three tunics, each

figure representing an average of ten measurements, are recorded in table 2. These figures substantiate the opinion obtained by examination of gross specimens and photomicrographs (pls. 2 and 3), namely, that the wall of the opossum gall bladder is appreciably thinner than that of the cat. The same holds for the tunica muscularis. Indeed, the width of the muscular layer in the collapsed gall bladder of the opossum is no thicker than the corresponding layer in the distended gall bladder of the cat (figs. 8 and 11). It would thus appear that the muscle tunic in the former would be less effective than in the latter species.

TABLE 2

*Measurements of microscopic sections*

| | ENTIRE WALL | TUNICA MUCOSA | TUNICA MUSCULARIS | TUNICA SEROSA |
|---|---|---|---|---|
| A. Distended gall bladder | | | | |
| Cat no. 25 | 0.437 mm. | 0.238 mm. | 0.087 mm. | 0.112 mm. |
| | 100% | 54% | 20% | 26% |
| Opossum no. 9 | 0.216 mm. | 0.135 mm. | 0.045 mm. | 0.036 mm. |
| | 100% | 62% | 21% | 17% |
| B. Collapsed gall bladder | | | | |
| Cat no. 26 | 0.774 mm. | 0.518 mm. | 0.153 mm. | 0.102 mm. |
| | 100% | 69% | 19% | 12% |
| Opossum no. 19 | 0.559 mm. | 0.416 mm. | 0.081 mm. | 0.061 mm. |
| | 100% | 74% | 14% | 12% |

Another anatomical feature which might affect the flow of cystic bile in the opossum is the shortness of the small intestine—a tube which consists of only three or four closely wound coils. Since Bronner and Schueller ('30) have shown that food does not activate the human gall bladder appreciably after it traverses the middle jejunum, it is quite possible that absorption from the relatively shorter intestine of the opossum is not sufficiently prolonged to induce sustained emptying of the gall bladder. Some support for this view is obtained by comparing the duration of the principal period of discharge of cystic bile in the swo species (table 1, a and

1, b): the average for the twelve opossums (101 minutes) being much less than that for the twelve cats (177 minutes). But this factor does not explain the striking variation within a given species, witness the great disparity in emptying-time and rate of discharge in the first and second group of cats; nor does it explain the initial delay in activation of the opossum gall bladder, as compared with that of man. Accordingly, we cannot escape the conclusion that the anatomical peculiarities described above are insufficient in themselves to explain all the physiological differences between various species or even within the same species. There remains, therefore, the hypothesis recently advanced by Doctor Boyden, in connection with his study of the primate gall bladder, that differences in reaction to food manifested by gall bladders of different species are primarily differences in physiological organization and depend upon factors that underlie the stimulation of the gall bladder or the regulation of the flow of bile at the duodenal orifice—such factors, perhaps, as the rate of emptying of the stomach, the rate of gastric and upper intestinal peristalsis, variation in absorption from the intestine, and the like.

## CONCLUSIONS

In a comparative cholecystographic study of the evacuation of the gall bladder in cats and opossums marked differences were noted in the reaction of the two species to a meal of egg yolk and cream.

The twelve cats exhibited two distinct methods of evacuating the gall bladder, seven of them discharging more than half the contents within forty-five minutes after the cystic duct begins to fill (in which respect they resemble the human gall bladder), and five emptying very slowly over a long period of time. At expiration of the period of contraction, the halogenated oil was completely evacuated in about five cases and only a small drop remained in the other seven.

The twelve opossums, on the other hand, consistently exhibited incomplete emptying of the gall bladder, rarely more

than half and usually less than a third of the contents. Not only was there an initial delay (an average of eighteen minutes elapsing before oil entered the cystic duct), but the principal period of discharge was limited to an average of 107 minutes, as against 177 minutes in the twelve cats. These striking differences in the reaction of the gall bladder of the opossum to food are only partially explained by such anatomical differences as the greater size of the gall bladder, its thinner muscular tunic, its tenuous cystic duct, the extramural position of the ampulla of Vater (the latter exhibiting marked peristalsis), and a reduction in absorptive area of the small intestine.

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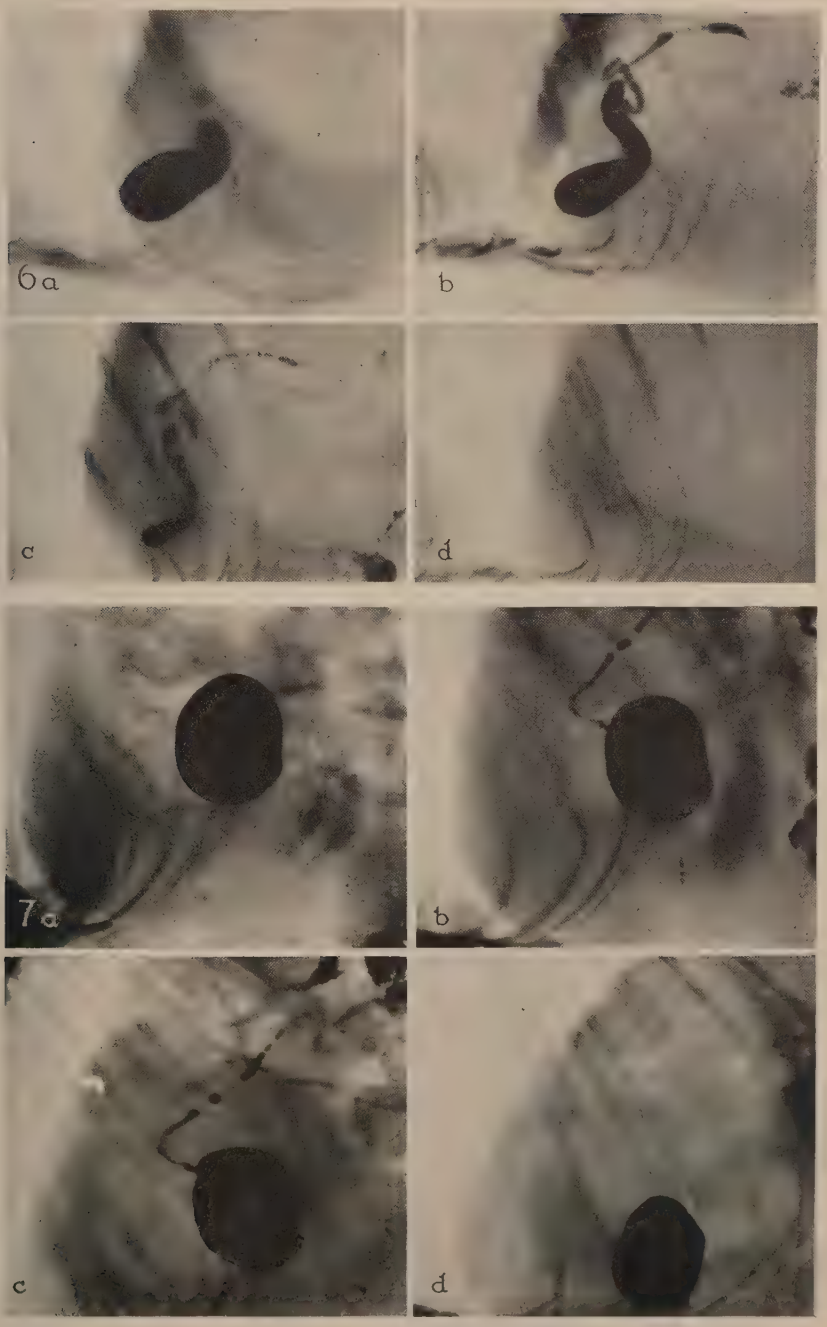
## PLATE 1

EXPLANATION OF FIGURES

6 Selected cholecystograms of cat no. 27 (compare table 1, a and fig. 2), illustrating discharge of opaque oil subsequent to meal of egg yolk and cream. × 7/10. *a*. Visualization of gall bladder three and one-half minutes preceding meal (thirteen hours after light brominol was placed in the gall bladder). Note small droplet of oil in cystic duct. *b*. Thirty-six and one-half minutes after egg yolk and cream. Note marked change in shape of gall bladder and passage of oil through tortuous cystic duct, common duct, and duodenum. *c*. One hundred sixty-two and one-half minutes after egg yolk and cream. Size of gall bladder considerably reduced. *d*. Five hundred thirty-two and one-half minutes after egg yolk. The last roentenogram to demonstrate oil in the gall bladder.

7 Selected cholecystograms of opossum no. 12 (compare table 1, b and fig. 3), illustrating discharge of opaque oil subsequent to meal of egg yolk and cream. × 7/10. *a*. Visualization of gall bladder three minutes preceding meal (fourteen and one-quarter hours after lipiodol was placed in gall bladder). Note the distinctly globular form of the vesicle. The small nubbin on the right of the viscus was produced by ligation subsequent to replacement of bile by lipiodol. *b*. Thirteen minutes after egg yolk and cream. Oil in cystic duct. Shape of gall bladder only slightly altered. *c*. Thirty-one minutes after egg yolk and cream. Further slight changes in shape of gall bladder. *d*. Five hundred seventy-four and one-half minutes after egg yolk and cream. Expulsion of opaque substance has ceased. Gall bladder has reverted to a shape comparable to that before feeding.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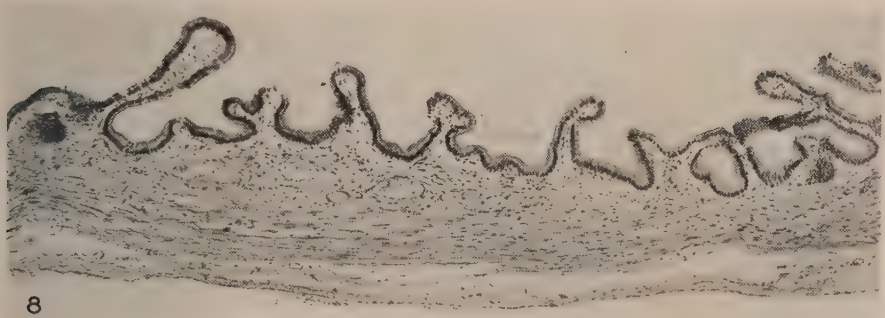
## PLATE 2

EXPLANATION OF FIGURES

Photomicrographs of the cat.

8 Cross section of gall bladder physiologically distended with bile (cat no. 25; hematoxylin and eosin; × 89; 10 μ). Note the low but distinct rugae and well-defined tunica muscularis. The muscularis in this specimen constitutes approximately one-fifth of the entire wall.

9 Cross section of collapsed gall bladder; five hours after a meal of egg yolk and cream (cat no. 26; × 89; hematoxylin and eosin; 10 μ). Note high bulbous and edematous plicae. The entire wall of this specimen is approximately twice as thick as that illustrated in figure 8.



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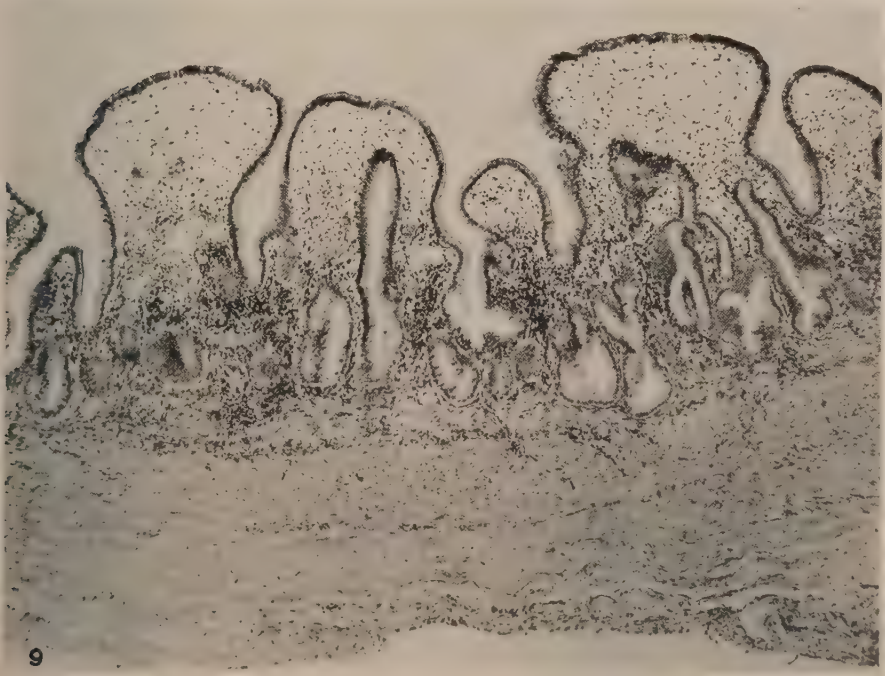

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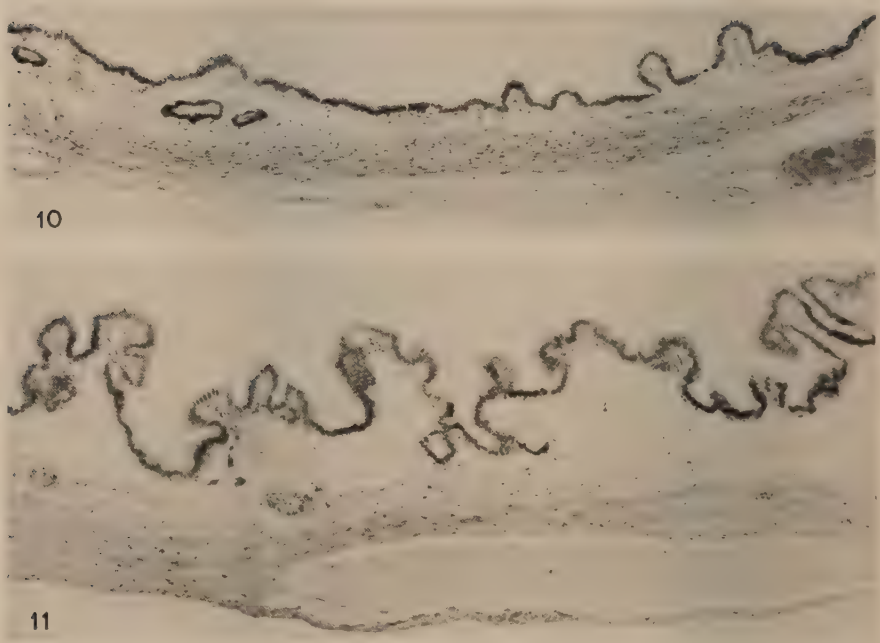


Photomicrographs of the opossum.

10 Cross section of gall bladder physiologically distended with bile (no. 9; hematoxylin and eosin; × 89; 10 μ). Note that rugae are nearly obliterated.

11 Cross section of a collapsed gall bladder (less than 0.5 cc.) six hours after a meal of egg yolk and cream (no. 19; hematoxylin and eosin; × 89; 10 μ). From a histological standpoint, the gall bladder of the opossum may be distinguished from that of the cat by its simpler pattern. Apparently the rugae are less folded, are not usually telescoped and the basal crypts are less branched. Also, under distention the rugae are flattened more than in the cat.

# THE REACTION OF TISSUE-CULTURE CELLS TO BARIUM (X-RAY) SULPHATE

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FOUR TEXT FIGURES AND ONE PLATE (TWO FIGURES)

In testing out the effect on the cells of the body of various substances used in chemotherapy, we are including in our studies barium (x-ray) sulphate, since in its general use in x-ray work it comes in direct contact with the cells of the digestive tract. It is chemically an inert substance and is supposed to be non-toxic to the cells of the body. However, in a recent paper Andrews and Paulson(1) have shown that the ingestion of barium sulphate by man resulted in a reduction in the number of intestinal protozoa per volume unit of the stool. This reduction in number, they think, may be due to the dilution of the contents of the intestine. We were curious to know whether it had any direct effect on the cells of the body and have performed the following experiments with tissue cells of chick embryos grown in vitro.

## MATERIAL AND METHOD

Hens eggs were incubated from seven to eleven days, the number of days depending on the tissue to be studied. They were then opened aseptically in small Petri dishes containing Locke-Lewis solution(2) (Locke solution [NaCl, 0.9 per cent; $CaCl_2$, 0.025 per cent; KCl, 0.042 per cent; $NaHCO_3$, 0.02 per cent], 85 per cent; chicken bouillon, 15 per cent; dextrose, 0.25 to 1 per cent). The tissues to be grown were removed, cut into small pieces about 1 mm. long, and mounted in hanging drops on cover slips which were inverted over

depression slides and sealed with hard vaselin. These explants were incubated at 35° until the growth of cells was sufficient for experimentation. The rate of growth varied with the particular kind of tissue, some tissues growing more rapidly than others. There was also some variation in the rate of growth of the same kind of tissue. The following table shows the age of the embryo and the age of the cultures of the various tissues used for the experiments.

| TISSUE | AGE, IN DAYS, OF EMBRYO | AGE, IN DAYS, OF TISSUE CULTURE |
|---|---|---|
| Intestine | 8 | 2, 3, 4, 5, 6, 7 |
| Kidney | 8, 9 | 2, 4, 5, 6 |
| Liver | 7, 8 | 4, 5, 6 |
| Lung | 8, 9 | 2, 3, 6, 7 |
| Muscle | 8, 9 | 2, 3, 4 |
| Retina | 8 | 3, 4 |
| Spleen | 11 | 3 |

In a former study (Hogue(3)) we found it advisable to add the solution which we were studying to a good growth of tissue-culture cells rather than to put the embryonic tissue into the solution and study its growth there, since there were frequently explants of tissues which did not grow at all. By using only good growths of tissue-culture cells, we knew what the effect of the substance was on them.

Barium (x-ray) sulphate was made up aseptically in Locke-Lewis solution in dilutions 1 to 1000. This gave a suspension of fine granules which floated evenly in the Locke-Lewis solution. This will be referred to as barium-sulphate suspension. There was no agglutination of granules, and the granules did not all settle to the bottom of the hanging drop. Many granules remained floating in an even suspension throughout the drop, so that the tissue cells migrating out from the explant came directly in contact with them. In adding the suspension of barium sulphate to the tissue culture the Locke-Lewis solution in which they had been growing was first withdrawn by a sterile pipette and then a platinum loop full of the barium-sulphate suspension was put over the

tissue-culture cells. After ten to twenty seconds the cover glass was again inverted over the depression slide and sealed with hard vaselin, the whole procedure being done aseptically. The medium was changed every two or three days. In this way the cultures were kept alive for days (twelve to fifteen, depending on how old the cultures were when the barium-sulphate suspension was added).

Controls were run for all experiments. These consisted of tissue cultures made at the same time and from the same tissue as those used for the experiments. They received fresh Locke-Lewis solution at the same time the experimental slides were receiving the fresh barium-sulphate suspension.

## EXPERIMENTS

### *Fibroblasts*

In the cultures of tissues grown from the intestine of an eighth-day-old chick embryo there were always large growths of fibroblasts. Some of those near the explant formed a reticular membrane, while those which had migrated farther out were solitary. When the suspension of barium sulphate was added to the tissue culture a few granules would remain sticking to the fibroblasts in the membrane. However, it was the outer fibroblasts, which were free-moving and migrating, which came continually in contact with the floating granules and with those which were adhering to the cover slip.

When a fibroblast was near one of these granules it would send out a long slender process which, when it reached the granule, usually stuck to it. If it stuck to the granule the granule would move slightly. More cytoplasm would flow into the process and it would quickly surround the granule closely without the formation of a vacuole. As the cell advanced more cytoplasm flowed into the process, making it larger, until soon the granule was in the body of the cell. In the meantime new processes were being sent out and other granules were being picked up and taken in until the cell contained many granules, some of them alone, others in groups (fig. 6).

Not all the granules approached by the fibroblasts were taken in. Sometimes the process would be withdrawn and the granule left free in the medium. At times it was difficult to see the fine ends of the processes of the fibroblasts, but we could always tell when they had touched the granule by the gentle oscillating movement of the granule.

The granules did not seem to affect the cells when they were taken in small amounts but when large numbers of granules were taken in, the cells would contract suddenly, sometimes leaving long, fine threads of cytoplasm (fig. 6, the two lower cells) which were later withdrawn, and the cells appeared as masses of granules with almost no cytoplasm visible. They would remain dormant for a few minutes, then they would begin sending out processes like blebs. Sometimes it took hours before these overloaded cells would readjust themselves and begin migrating again. Some fibroblasts did not recover. After sending out blebs for several hours, they became less and less active and finally died. The length of life of these cultures is similar to that of the controls, though at times the rate of growth seemed slightly slower.

One fibroblast which had taken in several granules began extruding its nucleolus. The nucleolus rose up as a small hill above the cell and in half an hour it had been squeezed out of the cell. It became wrinkled. The cell contracted and soon died.

These migrating cells really cleared the space around them of granules. The field under the microscope would be evenly covered with granules, except around these loaded cells which had taken in all the granules in their immediate neighborhood.

In addition to the granules inside the cells, there were usually many granules sticking to the outside of the cell. Some of these granules became attached to the cell when the suspension of barium sulphate was added to the culture. Other granules were partially ingested as the cells migrated. Sometimes only a small part of the granule would be attached to the cell and the granule would move back and forth as the cell moved (fig. 1). We followed these partially ingested granules

for hours. The cell trailed them along at the end of a long process, eventually the process carrying its granule was withdrawn into the body of the cell, the granule still remaining only partially ingested.

Two fibroblasts containing a few barium-sulphate granules were watched as they divided. Some of the granules went to

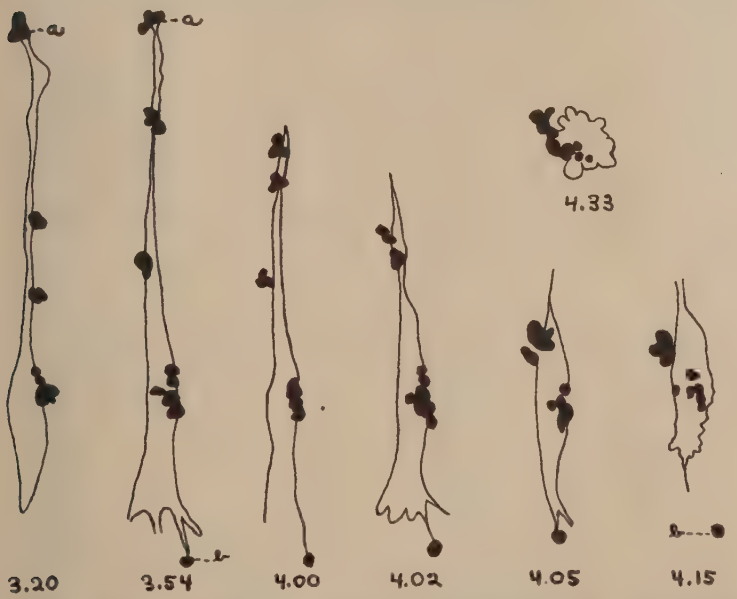


Fig. 1 Partial ingestion of a barium-sulphate granule (*a*) by a fibroblast. The cell contracts and draws its posterior process into the body, though the granule still remains only partially ingested. Another granule (*b*) is set free when the cell draws in its anterior processes and dies. Camera-lucida drawing, 4-mm. Obj., 10 oc.

each daughter cell. The presence of these granules did not seem to affect the mitosis.

We studied fibroblasts from explants of the intestine, kidney, liver, lung, skeletal muscle from the leg, spleen, and retina. They all behaved in the same way, taking in granules of barium sulphate, the outermost cells of the growth loading themselves down with the granules.

*Macrophages*

Macrophages appeared rather late in most of our tissue cultures. To some three-day-old cultures of intestine and of liver, we added barium-sulphate suspension. Nine days later, we found the macrophages loaded with granules. It was very easy to follow the ingestion of the granules by the macrophages. Their processes were not so fine and not so thin as

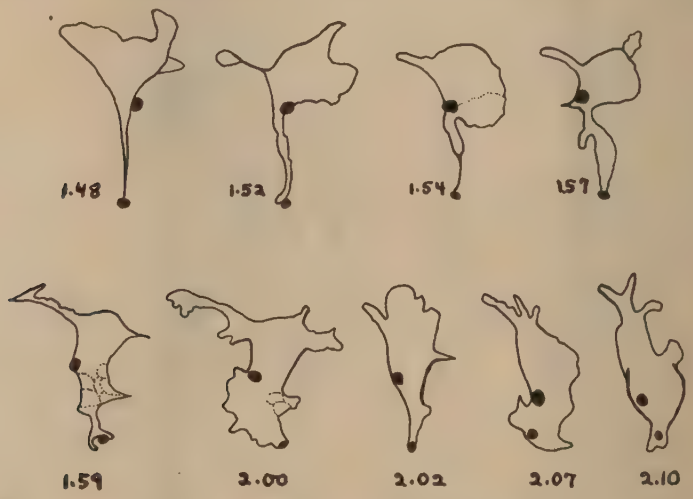


Fig. 2 A macrophage ingesting a barium-sulphate granule. At 1: 48 it touches the granule with its long slender process. This process becomes broader and foam-like and surrounds the granule (1: 59) which is then taken into the cell (2: 10). Camera-lucida drawings, 4-mm. obj., 10 oc.

those of the fibroblasts, so that the ends could readily be observed. They, too, sent out long processes which touched the granule (fig. 2), surrounded it closely, and then became a broad foam-like process by the addition of more cytoplasm. This process with its granule was then withdrawn into the body.

*Epithelium*

The epithelial cells of the intestine grow out in membrane formation. They are small cells, closely packed together. At

the outer free surface of the advancing membrane the epithelial cells send out slender processes. In older cultures (five or six days old) the outer edge of the epithelial plate is often covered with fibroblasts, but these can easily be distinguished from the epithelial cells, as the fibroblasts are larger and have larger nuclei and their processes are more irregular and come from any part of the fibroblast, while in the epithelial cell they come only from the advancing free surface. A single epithelial cell would often send out several slender processes. One of these processes, often at the expense of the others, would advance until it came in contact with a granule which it usually surrounded with cytoplasm. This was then drawn back into the body of the cell and new processes were sent out which picked up more granules.

The effect of this loading up of the outermost epithelial cells with granules was very spectacular. It gave the appearance of a heavy black ring of granules around the edge of the epithelial plate (fig. 5). Sometimes these plates of epithelial cells growing out from different parts of the explant would meet and become one continuous sheet of cells. In these cases the cells at the sides with their granules would then become embedded in the body of the plate and appear as black lines of granules radiating out from the explant. This appeared only in older cultures where there were large plates of epithelium.

The epithelial cells from the intestine, liver, retina, and from the tubules of mesonephros and from the alveoli of the lung, all grow out in plate formation. In each case the outermost cells of the plate take in the barium-sulphate granules. The cells do not seem to be disturbed by the presence of these foreign bodies.

In the tissue cultures from the mesonephros we sometimes found giant cells. These contained many nuclei and were always found loaded with barium-sulphate granules.

*Skeletal muscle*

For the experiments with embryonic muscle cells from the leg we used only good growths containing long multinucleate cells with their ends free from fibroblasts and with many fine processes. When the barium-sulphate suspension was added to these cultures, we followed the taking in of the granules by the fine processes in the same manner that the fibroblasts and epithelial cells had taken them in (fig. 3). Later, the

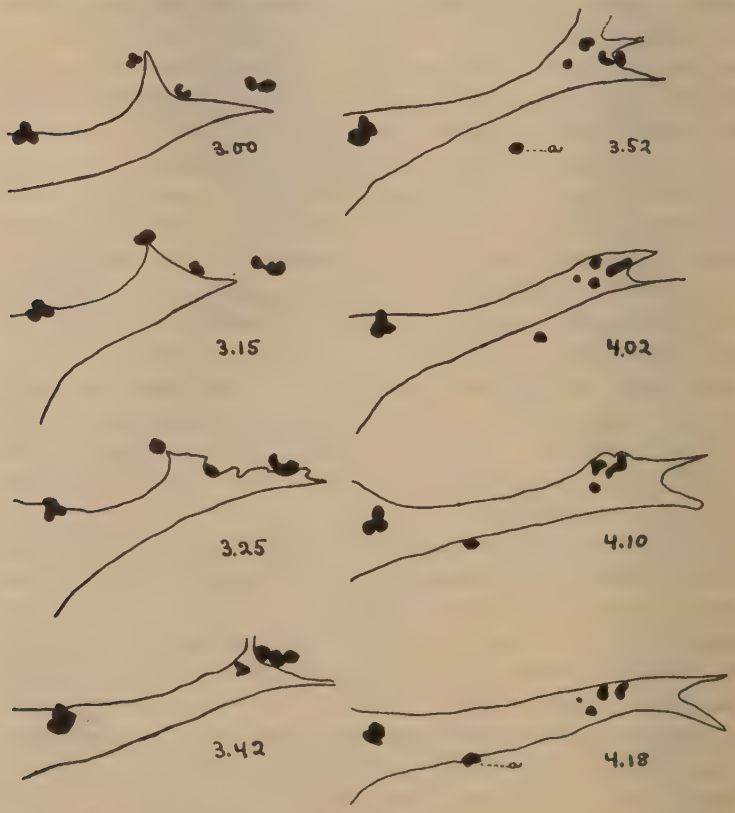


Fig. 3 The end of an embryonic skeletal muscle cell grown from the leg of a chick embryo eight days old. When barium-sulphate suspension was added, the end of the muscle cell sent out processes which surrounded and took in four granules. At the lower side of the cell a small particle is shown which approaches the side of the cell and sinks part way down in it, i.e., is partially ingested by it. Camera-lucida drawing, 4-mm. obj., 10 oc.

slide was fixed in Susa solution and stained with iron haematoxylin. The same cell was studied again to be sure the granules were inside and not merely sticking to the outside. Not all the granules were taken in. Often the muscle cell would glide slowly past them as it grew out into the medium. Some cells seemed more ready than others to take up the granules. Many muscle cells had granules also sticking to their surfaces. Figure 3 also shows a granule (*a*) which drifted over to the muscle cell. It touched the cell at 4:10 and then granually sank in, becoming partially ingested by the cell.

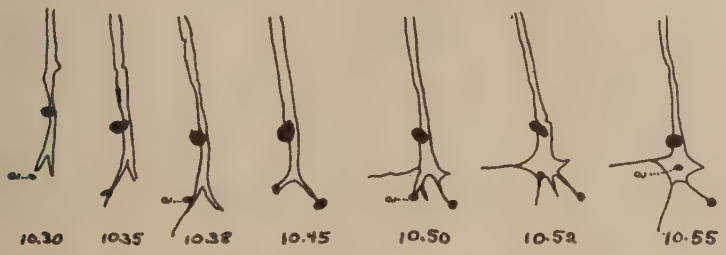


Fig. 4 A sympathetic nerve ending growing out in barium-sulphate suspension. At 10:30 it is sending out a process which in later stages touches a granule (*a*) and draws it back into the nerve ending. Camera-lucida drawings, 4-mm. obj., 10 oc.

*Nerve*

The sympathetic nerves from the plexuses of the intestine grew out in many cultures. When barium-sulphate suspension was added to these cultures, many granules would stick to the nerves. One case was followed where a fibroblast, passing over a nerve, took from it a granule. The granule continued to adhere to the surface of the fibroblast as it moved along. However, as a rule, the granules stuck to the sympathetic nerves.

The ends of these sympathetic nerves have very fine protoplasmic processes which go out in all directions as the nerve grows out from the explant. We followed these processes as they came near granules (fig. 4). To our surprise, they, too,

sent out longer and longer processes which surrounded the granule and later drew it back into the flattened plate-like ending of the nerve. Since the nerve with its ending is a part of the nerve cell, we have here nervous tissue ingesting foreign particles.

## DISCUSSION

### *Attraction of foreign bodies for cells*

The reaction of living cells to foreign particles has been the subject of innumerable studies with many kinds of particles and many kinds of cells. We will not attempt here to review this voluminous literature, but simply refer to a few studies which are particularly pertinent to our work. Smith, Willis, and Lewis(4), using tissue-culture cells and tubercle bacilli, found "that the cell did not make any active movements toward the bacillus, nor was any migration of bacilli toward the cell observed." On the other hand, Fenn(5) has observed a great difference in the reaction of leucocytes to $MnO_2$ particles and Mn silicate particles. He says:

> there are 2.4 times as many contacts per unit time with $MnO_2$ as with the Mn silicate although present in equal numbers. Here there is evidently something like a stimulus which comes from the $MnO_2$ particles and causes an extra liberation of energy from the leucocyte resulting in an apparently purposeful progression in a definite direction and prompt ingestion.

In our experiments we cannot attribute any selective action to the cells. They ingested the granules in their pathway as they moved outward from the explant. Some cells did not ingest certain granules, but passed them by. Other cells must have ingested them, as eventually the whole field around the explant was cleared of granules and the cells were loaded with them.

### *Method of ingestion*

Smith, Willis, and Lewis(4), in studying the ingestion of tubercle bacilli by tissue-culture cells, say that: "the only bacilli that were taken in by the cells were those which came

in direct contact with the cytoplasm throughout their entire length.'' They also say that ''the cells did not send out processes around it'' (the bacillus). In the case of the macrophages, they describe large ''sheet-like processes which furl and unfurl down into the medium . . . . Despite these numerous processes, none of these cells was seen to 'take in' a bacillus by means of them.''

Smith(6) describes the taking in of the melanin-pigment granules ''as a gradual sinking of the granule into the cytoplasm. This . . . . is illusory, as the granule entered the cytoplasm from the under surface of the cell and could not sink upward.'' He concludes that they must be taken in by capillary attraction, since he observed no formation of protoplasmic processes.

Mudd and Mudd(7) have observed in vitro the reaction of leucocytes and macrophages to oil droplets, to specifically sensitized bacteria, and to red blood cells. The oil droplets 'sink into' the macrophages without the formation of any processes. On the other hand, the polymorphonuclears and occasionally the macrophages flow over clumps of specifically sensitized bacteria and red blood cells by means of more or less blunt projections of the cell cytoplasm.

Our observation on the reaction of tissue-culture cells to barium-sulphate granules was somewhat different from these. We did see the partial ingestion of granules by the 'sinking in' method. Whether they eventually were completely ingested, we do not know. They seemed to remain partially free and able to move back and forth as the cell moved. We did, however, see all these tissue-culture cells forming definite processes which went out to the granules, surrounded them, and drew them into the body of the cell.

From all these observations we can only conclude that cells react differently to different kinds of particles. We agree with Mudd and Mudd that much depends on the nature of the particle, the condition of the cell, and the conditions of the observations.

### *Digestion of particles*

Smith, Willis, and Lewis(4) described the digestion of the tubercle bacilli by the tissue-culture cells, which in brief was: "a small vacuole formed about the microorganism which was eventually destroyed." Smith(6) describes the later formation of vacuoles around the melanin-pigment granules and the partial or complete digestion of these granules in practically all the cells studied. In the material we used we never saw the formation of vacuoles. When the barium-sulphate granule was taken in, the cytoplasm closely surrounded the granule. Nor did we later see the formation of digestive vacuoles. The particles as seen in stained preparations are finely granular and of even size. This is probably because only small particles were taken in. They were not digested and we did not see them extruded as we observed in the case of melanin-pigment granules taken in by amoeba living in tissue cultures (Hogue(8)). Cells which had been in the barium-sulphate suspension for several days still retained the granules.

### *Which cells are phagocytic*

Smith, Willis, and Lewis(4), in their work with tubercle bacillus, say that "no microorganisms were observed in the red blood cells, striated muscle cells, nerve cells, or ciliated epithelial cells." Smith(6), working with melanin-pigment granules, reported that they were not taken in by muscle cells or sympathetic nerve cells. We were fortunate in seeing both the skeletal muscle cells and the sympathetic nerve cells take in barium-sulphate granules. We are, therefore, much interested in Lubarsch's(9) remark: "Ich pflege in meinen Vorlesungen auseinanderzusetzen, dass es keine Zellart (die Ganglienzellen und Knochenkörperchen ausgenommen) gibt die nicht gelegentlich als Phagocyten auftreten kann." We agree with this, but would extend it to include sympathetic-nerve cells, which we have shown have the power of taking in granules in their nerve endings.

## CONCLUSIONS

1. Barium sulphate did not affect the length of life of the cultures. They lived from twelve to fifteen days in the suspension, the time depending on the age of the culture when the suspension was added.

2. In some cases barium sulphate seemed to affect slightly the size of the growth. This was due to the overloading of the outer cells with barium-sulphate granules.

3. Barium-sulphate granules were ingested by fibroblasts from many parts of the embryo, by macrophages, by skeletal muscle cells, by sympathetic nerve endings, by giant cells of the kidney, and by epithelial cells from the intestine, retina, uriniferous tubules, lung alveoli, and liver.

4. There was no evidence of digestion of barium sulphate by the tissue-culture cells.

5. In all cases, except the sympathetic nerves, the cells sent out processes which closely surrounded the granules and took them into the body of the cell without the formation of vacuoles. In the case of the sympathetic nerve, the process sent out by the nerve ending surrounded the granule and drew it back into the nerve ending.

6. Partial ingestion of granules by their sinking into the cell was frequently seen, but their complete ingestion by this method was not observed. In this way granules adhered to many of the tissue-culture cells and to the nerve fibers.

7. Some fibroblasts became overloaded with granules and contracted. Some of these died. Others, after a period of rest, recovered.

8. Fibroblasts containing barium-sulphate granules divided forming two daughter cells, each of which contained granules.

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# PLATE

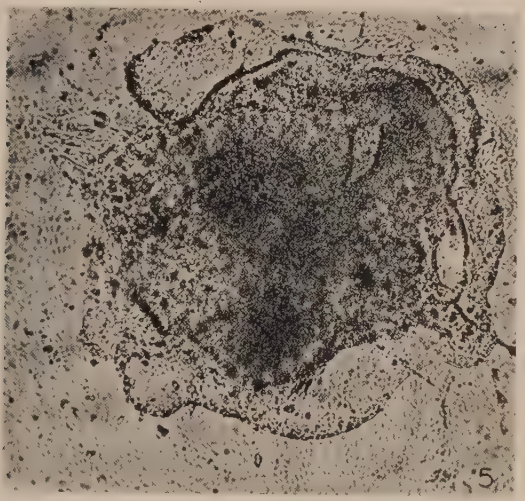
## PLATE 1

EXPLANATION OF FIGURES

Photomicrographs by B. B. Varian

5 Photomicrograph of a living tissue culture grown from the intestine of an eight-day-old chick embryo. Around the explant are plates of epithelium, the outer cells of which have ingested barium-sulphate granules. These granules form a black ring around the epithelial plate. The background is part of a larger plate of epithelial cells growing on the cover slip. × 53.

6 Photomicrograph of a living tissue culture of fibroblasts grown from a piece of muscle taken from an eight-day-old chick embryo's leg. Most of the cells have ingested barium-sulphate granules, which appear as black particles. Two cells in the lower part of the plate have contracted and show fine protoplasmic rays. × 250.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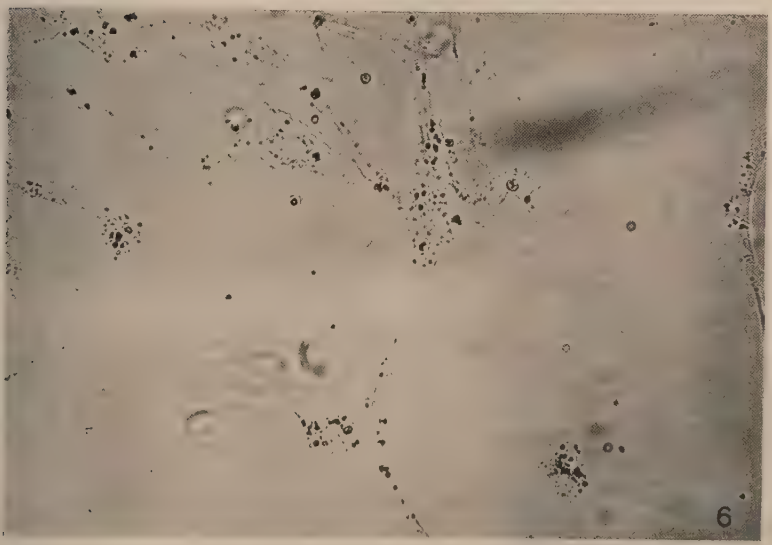
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# DELAYED PREGNANCY IN MICE

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THREE TEXT FIGURES AND ONE PLATE (EIGHT FIGURES)

Delayed pregnancy in mice is associated with the presence of suckling young. The simple state of affairs is that impregnated lactating females deliver their young not at the expected term, but from one to sixteen days later. Our first problem was to repeat the observations of other investigators (Pinard, '05; Daniel, '10; King, '13; Kirkham, '16, '18; Mirskaia and Crew, '30) in order to determine any possible correlation between the number of suckling young and the degree of delay. Secondly, we have attempted to ascertain the sequence of intra-uterine events responsible for the delay. Finally, our examination of living material has permitted the observation of the behavior of mouse embryos at the critical stage of implantation.

## I

The mice used in these experiments were of the Bagg-Little albino strain, and the foundation stock was supplied to us by Dr. E. C. MacDowell, of the Department of Genetics, Carnegie Institution of Washington.

Our experimental animals may be divided into three groups. The first consisted of a set of females kept continuously with males. Since the period of gestation in non-lactating animals of this strain is twenty days (compare MacDowell et al., '27), and since the birth date of every litter was carefully recorded, it was comparatively easy to determine delayed pregnancy. On the assumption that mating occurs at parturition, any pregnancy lasting from twenty to thirty-five days after a pre-

vious parturition is taken as delayed. Births occurring at forty or more days after a previous parturition are excluded as probably normal; we have no records of births occurring at thirty-six to forty days after a previous parturition. The births recorded as delayed might have been due, in part, to superfetation, to delayed fertilization, or to impregnation during lactation. We agree with King ('13) that these possibilities may be excluded, however, our general experience indicating that such cases must be extremely rare, if they occur at all.

In our second group of animals pregnant females of comparable age were isolated and examined twice daily for litter births. As soon as a litter was observed a male was placed with the female for twelve hours. The presence of a vaginal plug was taken as evidence of mating.

The third group of mice were mated in the same manner as the animals of our second group, but the females were killed and dissected at various times during the second pregnancy.

The uteri were removed, placed in Ringer's solution, and cut free of the mesovarium, ovaries, and tubes. In most cases the ovaries were examined for recent corpora lutea and in some cases fixed and sectioned. The uteri, freed of adherent Ringer's solution by being placed on filter paper, were weighed, and then one horn was flushed with Ringer's solution to wash out any blastodermic vesicles that might be present. If the washings were clear the uterus was slit open lengthwise, and examined under a dissecting microscope for implantations. Blastoderms present in the washings were taken up by a special micropipette with a fine capillary and a bulbous portion covered by a rubber dam. The vesicles were transferred to a moist chomber for observation and a number were photographed.

The second horn of the uterus (or embryos of large size) was fixed either in Bouin's fluid or Maximov's modified Zenker's fluid. Sections cut at 10 to 14 μ were stained in Delafield's hematoxylin and eosin.

II

In figure 1 we collect the data from our three groups of animals. The calculated delay in the animals killed before parturition is based on the following considerations. First, the growth curve of implanted embryos in these females is the same as the growth curve of embryos in non-lactating females. We have accepted this proposition because: 1) the birth weight of young from delayed parturitions is the same as the birth weight of young born at the normal term; 2) we have compared growth weights of implanted embryos from normal and from delayed pregnancies, and we find no difference if the implantation date is taken as the beginning of the growth curve. Secondly, we have used MacDowell's ('27) standard curve for mice and find our data on normal and delayed embryos to check surprisingly well with his. Thirdly, we have omitted in our table any calculations based on the free blastocysts, as we have not found any reliable criterion of relative blastocyst age in delayed pregnancies. Finally, we have employed morphological criteria as to the relative age of the implants, by comparing sectioned embryos from delayed pregnancies with normal embryos, after the manner of Kirkham ('16). Unfortunately, the application of Kirkham's table to our data is vitiated by the fact that the normal period of gestation in his mice was twenty-two days as compared with twenty days in our strain, so that we have found relative weight of embryos a better criterion than the morphological indices of development. From figure 1 we can deduce that each suckling young delays the ensuing parturition by twenty-one hours, rather than one day (compare Daniel, '10).

Our data show a slight positive correlation between the number of young carried and the degree of delay of pregnancy. The relation, however, is in no sense marked, and we attribute to it no causal significance. Obviously, if the number of young carried affected the length of the pregnancy, non-lactating females should also show delayed pregnancies, and this is not in fact the case.

We find also a very slight relation between the weight of a female at parturition and the length of the succeeding pregnancy. Eight females showing no delayed pregnancy weighed 28.6 ± 0.6 grams; six who showed a delayed of one to five days weighed 28.5 ± 0.6 grams; eight who showed a delay of five to ten days weighed 27.7 ± 2.8 grams; and a group of fourteen who produced no young, either because of failure of ovulation or implantation, weighed 27.2 ± 0.6 grams.

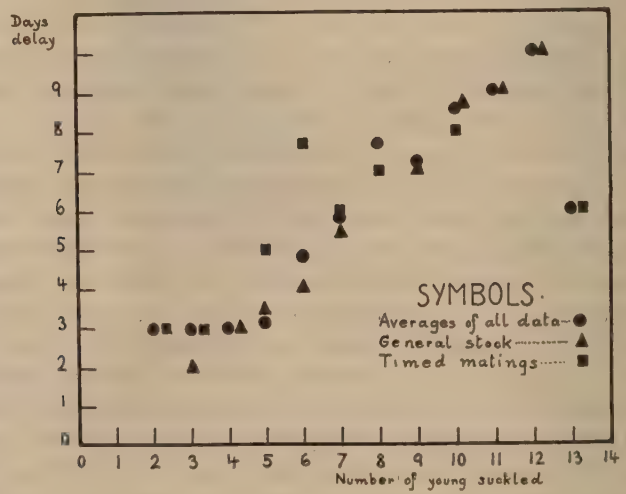


Fig. 1 The relation between the number of suckling young and the days of delay of pregnancy in three series of mice. (See text.)

III

Kirkham ('16, '19) finds that lactating mothers either fail to ovulate at parturition, or, if ovulation and fertilization occur, the implantation of the blastocyst is delayed until the fourteenth day after mating. We have killed and dissected fifty-three females with suckling young from three to twenty-one days after mating at parturition and have recovered embryos in twenty-nine of the animals. It is highly probable that in some of our twenty-four negative cases no ovulation occurred. Furthermore, our data establish that a delay in

implantation does occur in these lactating females. Kirkham's observations are therefore confirmed, but certain qualifications must be put upon his general propositions. These will be evident from an examination of table 1.

The finding of free blastocysts at any time until the eighteenth day after mating and of implanted embryos as early as the tenth day may occur not after a nine day's delay

TABLE 1

*Summary of findings on lactating females dissected at various times after mating*

| TIME BETWEEN MATING AND DISSECTION, DAYS | NUMBER OF FEMALES WITH FREE BLASTOCYSTS | NUMBER OF FEMALES WITH IMPLANTED EMBRYOS | NUMBER OF NEGATIVE CASES |
|---|---|---|---|
| 3 | 1 | .. | .. |
| 4 | 1? | .. | 2 |
| 5 | 2 | .. | .. |
| 6 | 2 | .. | 3 |
| 7 | 1 | .. | .. |
| 8 | 2 | .. | 2 |
| 9 | 3 | .. | 5 |
| 10 | .. | 1 | .. |
| 11 | .. | .. | 2 |
| 12 | 1 | .. | 2 |
| 13 | 1 | .. | 2 |
| 14 | 1 | .. | 1 |
| 15 | 1 | .. | 1 |
| 16 | 1 | .. | .. |
| 17 | .. | 2 | .. |
| 18 | 1 | 3 | 2 |
| 19 | .. | .. | 1 |
| 20 | .. | 1 | 3 |
| 21 | .. | 2 | .. |

as Kirkham states, but at any time over a period of days. A careful examination of the degree of development of the blastocysts observed indicates that implantation may occur in lactating animals between the sixth and sixteenth day after mating. In view of our findings in the preceding section, it is evident that the time of implantation will depend on the number of suckling young.

The set of blastocysts recovered at eighteen days after mating showed definite signs of degeneration (fig. 9). We

believe that these would not have become implanted. It seems probable, therefore, that sterility in some lactating mothers may be due to failure of implantation as well as to failure of ovulation.

Implantation in non-lactating mice occurs at the sixth day after mating (Sobotta, '95, '03, '08, '11; Jenkinson, '00; Burckhard, '01; Melissinos, '07; Long and Mark, '11; Long, '11; Kirkham, '16, '19, '20, and others). It therefore follows that the blastocysts in our lactating females may remain free

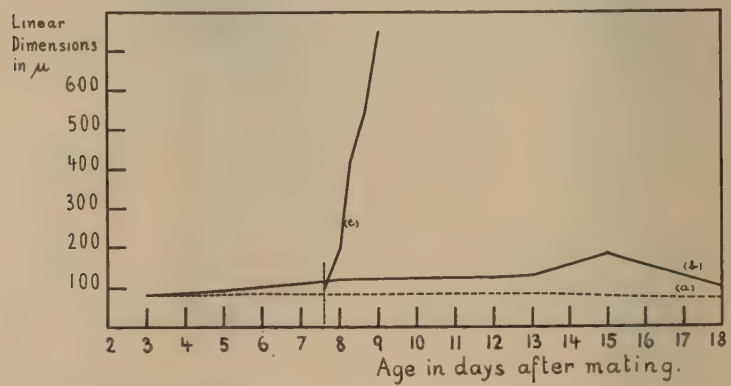


Fig. 2 Growth of early embryos: *a*) width, unimplanted blastocysts; *b*) lenth, unimplanted blastocysts; *c*) length, implanted embryos.

in the uterus as long as ten days after the normal time for implantation. Our observations indicate that they enter the uterus between the third and fourth day after mating, as in non-lactating mice and at the same stage of development. Furthermore, we find that the growth curve of the implanted embryos is exactly like that if implanted embryos in non-lactating females.[1] It would follow, therefore, that little or no growth or development occurs during the period of delay before implantation. Our direct observations indicate that this is the case. The data presented graphically in figure 2 demonstrate that extremely little growth of the blastodermic

[1] The detailed account of the growth curve will be presented in a later paper.

vesicles occurs during their prolonged sojourn as free blastocysts in the uterus, especially when one compares them with growing implants of the same age, even though no correction is made for shrinkage in fixed implants.[2]

It is true, of course, that a certain amount of development does occur in the blastocysts after they enter the uterus. This development parallels that of blastocysts in non-lactating animals up to the sixth day after mating. From the sixth day onward the blastocysts of lactating animals undergo at most but slight growth or differentiation. We have assembled in plate 1, figs. 4 to 12, a set of photographs of embryos recovered at various times after mating.

The blastocysts entering the uterus at about the third day after mating are much like normal embryos of the same age (compare fig. 4). The vesicle is perfectly round, consisting of a morula about 80 μ in diameter surrounded by a definite zona pellucida. According to Sobotta ('11), the zona pellucide disappears by this time. Our observations indicate that it persists till the end of the fifth day in both delayed and normal blastocysts (see also Widakowich, '09). By the fifth day the blastocysts have become ovoid in shape (fig. 6), with no change in width, but with an appreciable lengthening. They are definitely blastula with at least a partly formed inner cell mass. By the end of the fifth day the blastocysts are beginning to lodge in mucosal pits. A lateral constriction appears in the ovoid blastocysts. This constriction (figs. 7 and 10) marks the beginning of the formation of the 'Traeger,' which develops from one pole of the vesicle. When the vesicle is implanted its long axis lies perpendicular to the long axis of the uterus and in the plane of the mesometrium. The 'Traeger' points mesometrad and toward the excentric lumen of the pregnant uterus. In general, this constriction is the most obvious sign of the progress of differentiation of the free blastodermic vesicle.

[2] Data on implanted embryo were taken partly from our own sectioned material and partly from the published figures of Sobotta ('11).

This constriction is shown in all stages later than the sixth day after mating. In old vesicles (fig. 9) it is again observed, but less marked. It seems that in such vesicles the portion below the constriction (the ectoplacental cone) suffers the least changes at the onset of degeneration. Free normal blastodermic vesicles resemble in shape a balloon with a gondola, which is represented by the cone. The latter is of darker color and in older specimens consists of cells of unequal size. In living specimens the cone is readily distinguished by its darker color. The differentiation of the vesicles seems to stop short in all cases of the processes leading to the inversion of the germ layers to be described below.

Implantation leads to rapid and profound changes, the most obvious being the explosive growth. Implantation itself is a gradual process. Huber ('15) describes the process for the rat. In the mouse conditions are similar. The lumen of the non-pregnant uterus is thrown into many folds. The ova become lodged in folds along the border opposite the mesometrium. The spatial distribution of the ova in the long axis of the uterus is still a mystery. Huber finds in the case of the rat that the character of the uterine mucosa changes prior to implantation; the lining cells of the epithelium, which are ordinarily high, become cubical. The changes occur in sharply circumscribed patches where the ova are lodged. Gradually pits are formed, each one containing one vesicle. The pits deepen into funnels. Widakowich, Huber, and others claim that the blastodermic vesicle changes shape due to pressure by the surrounding space. The writers cannot agree with such a view. The change of shape from spherical to oval occurs gradually and begins even before the vesicles become lodged in pits. The changes in shape seem to be due chiefly to the development of the ectoplacental conus, the formation of the segmentation cavity, and 'Deckzellen' (covering cells).

The funnel-shaped decidual crypts (fig. 11) communicate with the lumen of the uterus. The ectoplacental cone finally plugs this opening. At this stage, the inversion of the germ

layers (entype) occurs. This was first reported for guinea-pigs by Reichert ('61). Since then it has been reported for other rodents by many observers. Entype consists in the forward pushing of the ectoderm into the endoderm until finally the ectoderm lies inside the endoderm. Sobotta ('11) describes this phenomenon as "canogenetische Anpassungsercheinung des Eies." He supposes that the inversion of the germ layers creates a larger surface for absorption and thereby makes rapid growth of the embryo possible. The germinal vesicle now becomes surrounded by lacunae filled with maternal blood. It grows by resorption of part of the decidua. Sobotta believes that the maternal blood is broken down and 'Haemoglobinschollen' are resorbed by the growing embryo. Our sections seem to bear out this view; they show vividly staining particles of red in the lateral folds of the growing amnion.

The vesicle is enclosed at this stage by a thin membrane, which has been called 'Reichert's membrane' by Melissinos ('07). This membrane is shown well in a whole mount of a living embryo (fig. 12). Melissinos describes the inner cell layer of the egg cylinder as consisting of three vesicles. Our observations agree with that of Sobotta ('11) and Widakowich ('09), who find two vesicles. Since we used living material, we have eliminated possible sources of error from shrinking due to embedding in paraffin. The final shape of the implant is the same whether pregnancy is normal or delayed. It is shown diagrammatically for the stage where the growth curve of the mouse embryo may be said to start, the time when the mesoderm is present.

## IV

Authentic data on delayed pregnancy in mammals are relatively scarce, and confined almost exclusively to rodents. Prolonged gestation in man has been described by Allen ('07) and similar cases in domestic animals by Göhring ('01) and Tessier ('17). Dr. R. Macdonald has shown us in his records a presumable case of delayed pregnancy in a whale. A fe-

male of Balaenoptera borealis was captured with her suckling calf in July, 1927. On examination, a foetus four feet long was discovered in the uterus. Since newborn calves in this species are usually about nine feet long, and parturition occurs normally in July (Harmer, '27), the foetus probably represented a delayed pregnancy.

For rodents alone are any quantitative data published. A definite positive correlation between the number of suckling

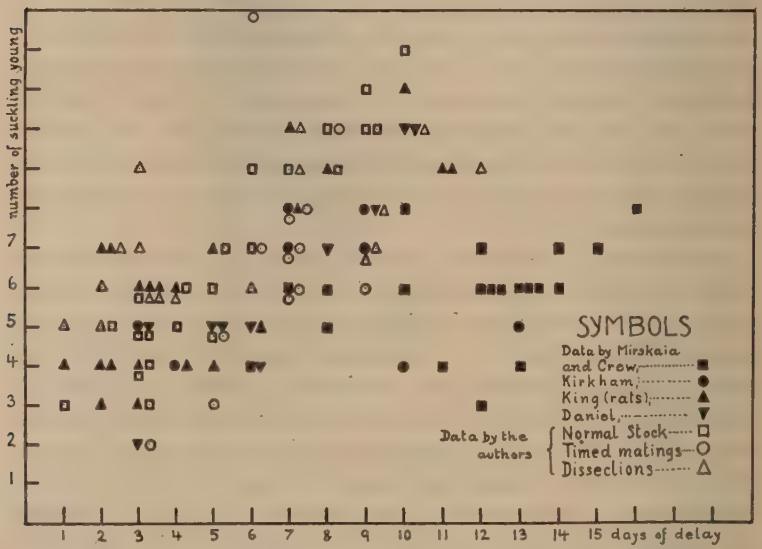


Fig. 3 The relation between the number of suckling young and the days of delay of pregnancy. All available data.

young and the number of days delay of pregnancy has been found for mice by Daniel ('10) and for rats by King ('16). Kirkham ('16) and Mirskaia and Crew ('30), working with mice, find no such correlation. As a matter of fact, the data of the latter writers do also show a slight positive correlation between number of suckling young and the degree of delay of pregnancy. We have grouped in figure 3 the data of all investigators, in spite of the slightly longer pregnancy (twenty-two days) of Miss King's rats.

It will be seen that our data and those of Miss King and Daniel fall quite well into the same grouping and indicate a delay of 21.4 hours for suckling young, whereas those of Kirkham and Mirskaia and Crew tend to fall together and somewhat below ours. These last two sets of data indicate, therefore, the same sort of relation that we find, the difference observed being possibly due to difference in the breeds of mice used.

Eaton ('32) finds in guinea-pigs no relation between the number of suckling young and the prolongation of gestation. His data indicate a positive correlation between the number of young carried and the degree of delay of pregnancy. All the available data on rats and mice indicate clearly that there is no regular relation in these species between the delay and the number of young carried. It is to be noted, moreover, that in guinea-pigs young born of delayed pregnancies are relatively heavier the greater the delay, whereas in mice and rats the birth weights of delayed and undelayed young are the same. It would seem, therefore, that the embryonic growth curve of guinea-pigs is affected by prolonged gestation. Eaton ('32) also finds that the age and physical condition of the dam are considerable factors in the guinea-pig. Our data are too meager to establish any definite relations to these factors in mice.

One consideration arising from our general examination is that any analysis of the intra-uterine growth curve of mice must distinguish between the preimplantation period and the postimplantation period. In the former period the blastocysts obviously receive very little nourishment from the uterine fluids, whereas as soon as implantation occurs a tremendous growth is begun, presumably because of the great increase in available nutrition. The growth of the early embryos in the tubes must also be comparatively slight. Gilchrist and Pincus ('32) have, in fact, shown for the rat that practically no increase in volume occurs in tubal ova. The analysis of MacDowell, Allen, and MacDowell ('27), where the formation of the embryo proper at implantation is taken

as the proper beginning of the growth curve, is therefore justified in contrast to that of Brody ('27), who uses the time of fertilization as starting point.

## SUMMARY

1. Delayed pregnancy in mice is related to the number of suckling young. Each young suckling prolongs gestation by twenty-one hours, on the average.

2. Delayed pregnancy is due to the failure of the blastodermic vesicles to become implanted at the normal time. Instead of becoming implanted at the sixth day after mating, as in undelayed pregnancies, the vesicles become implanted some time thereafter. Late implantation may occur from the seventh to the sixteenth day after mating; the greater the number of suckling young, the later the implantation.

3. Blastocysts that become implanted late show little or no development beyond that attained at the sixth day after mating in undelayed free blastocysts. As soon as implantation occurs, however, growth and development proceed at the same rate as in undelayed implants.

4. Lactating females may fail to produce any embryos. This may be due to failure of ovulation, or, in some cases, to failure of implantation. The sojourn of free blastocysts in the uterus beyond a certain period may lead to their degeneration.

5. The development of the blastocysts in the uterus of lactating females parallels that of blastocysts in non-lactating females up to the sixth day after mating. The implantation process in delayed implants is essentially like that of undelayed implants. A brief description of the preimplantation and implantation events is given.

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# PLATE

## PLATE 1

### EXPLANATION OF FIGURES

Photomicrographs of early mouse embryos. × 382.

4 Blastocyst recovered three days after mating of non-lactating female.

5 Blastocyst recovered five days after mating of non-lactating female.

6 Blastocyst recovered four and one-half days after hating of lactating female.

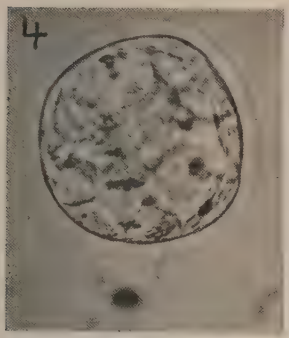
7 Blastocyst recovered thirteen days after mating of lactating female.

8 Blastocyst recovered fifteen days after mating of lactating female.

9 Blastocyst recovered eighteen days after mating of lactating female.

10 Embryo dissected from uterine wall at time of implantation. Recovered from non-lactating female early in the eighth day after mating.

11 and 12 Sectioned embryo from same female as in figure 10.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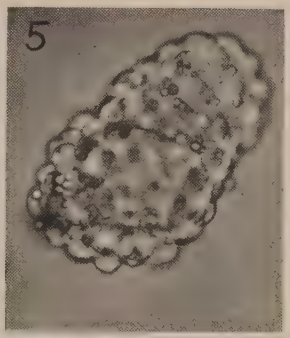
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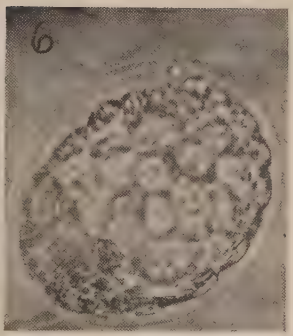
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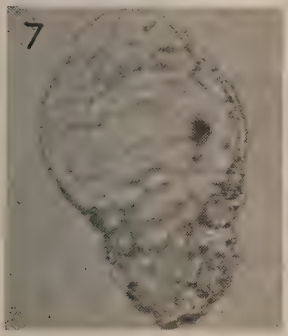
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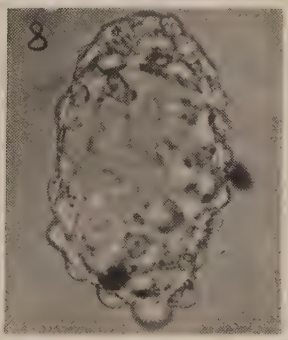
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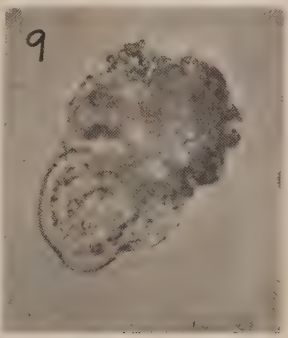
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# THE RELATION OF PREGNANCY CELLS IN THE PITUITARY OF THE RAT TO THE REPRODUCTIVE CYCLE[1]

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ONE PLATE (FOUR FIGURES)

Since the original report of Comte ('98), that the hypophysis undergoes definite changes during pregnancy, a number of papers have appeared dealing with the structural characteristics of the gland during the reproductive cycle, and Comte's observations on the human hypophysis were extended to, and confirmed in, lower forms by several subsequent workers. Included among these are Guerrini ('05), working on the dog and rabbit; Morandi ('05), Guinea-pig; Naegeli ('11), rat and guinea-pig; Pende ('11), cat; Schenk ('12, '26), rat; Kolde ('12), guinea-pig; Wittek ('13), cow; Berblinger ('14, '21), guinea-pig; Lehmann ('28), rat.

Erdheim and Stumme ('09) first called attention to the fact that, associated with the hypertrophy and hyperplasia described by Comte, there appears also a cell type of an atypical nature, constant in its occurrence and dissimilar in its nature to any type found in the non-pregnant gland; this was designated the 'pregnancy cell.' Subsequent investigators were in agreement that a distinctive cell type occurs and that the anterior pituitary in all forms studied undergoes a definite histological modification during the period of gestation—a modification which apparently occurs at no other time in the history of the gland.

[1] The expenses of this investigation were defrayed by a grant from the Bache Fund of the National Academy of Sciences.

The appearance of an invariable structural change in the pars anterior during pregnancy assumes added interest in view of recent developments regarding the pituitary-gonadal relationship, and it seems important that more concise data be acquired concerning the histological rhythm of the female hypophysis during the reproductive cycle of the animal. The present investigatoin is concerned with the temporal relations of the pregnancy cell type in the rat, i.e., the time at which these cells become evident, their persistence, and the influence of parturition and of lactation upon the modified pituitary of pregnancy. Adequate data bearing upon these points, so far as the author is aware, are completely lacking for the laboratory animal.

## MATERIAL AND METHOD

Virgin female rats were selected, a qualification which was emphasized, since it is not known at present what effect previous pregnancies might have upon the histological structure of the hypophysis. In the human being, at least, it appears that the histological picture is modified by previous pregnancies (Erdheim and Stumme, '09) in that pregnancy changes retrogress more slowly in multiparae. The animals were kept isolated, oestrous cycles were followed by the vaginal-smear method, and pregnancies were dated from actual copulation or from discovery of a vaginal plug. Material was obtained from, 1) a series of timed pregnancies; 2) a series of postpartum animals, sacrificed at varying intervals throughout the period of lactation; 3) a series of daily stages following premature withdrawal of litters; and, 4) a series of stages throughout pseudopregnancy.

In the first, or pregnant, series, animals were sacrificed at daily intervals throughout the gestation period of twenty-two days, at least three animals being used for each stage, a number subsequently augmented for the critical stages. In the postpartum series, six rats were sacrificed during the oestrous period which occurs twelve to twenty-four hours postpartum, and lactating females were sacrificed on the first,

second, third, and alternate days thereafter through the first postweaning oestrus. Pseudopregnant animals were secured by two methods; as a result of infertile copulation and through mechanical stimulation of the cervix, and were sacrificed according to the same routine employed for pregnancies.

Pituitaries, ovaries, and uteri were fixed in Zenker-formic-osmic fixative, embedded in paraffin, and sectioned serially. Pituitaries were sectioned at 3 μ, other tissues at 5 and 7 μ. All material was stained with Delafield's haematoxylin and eosin or with Mallory's triple connective-tissue stain. All instances of pregnancy were verified by examination of ovaries and uteri.

## HISTOLOGICAL OBSERVATIONS

### *The pituitary during pregnancy and parturition*

The anterior lobe recovered from animals during the first two days post coitus, and examined histologically, is indistinguishable from the non-pregnant gland except for slight indications of hyperemia (fig. 1).[2] The vascular network appears somewhat distended, particularly in the dorsal portion of the gland, i.e., adjacent to the pars intermedia. The cell masses retain their compact arrangement between the blood vessels. The three cell types, eosinophiles, with fine, closely packed granules, somewhat larger basophiles, and chromophobes, apparently occur in normal proportion.

The first appearance of pregnancy changes is somewhat variable. In a number of glands removed on the third day after copulation modifications are discernible as indicated by the presence of small scattered cells with a fairly large compact nucleus and a very meager amount of clear eosin-stained cytoplasm (eosinophil). In other instances, however, no changes of this nature are detectable. Pituitaries from pregnancies of four days' duration are uniformly modified, their vascularity being their most striking characteristic. The cell masses appear to be much more loosely aggregated,

[2] The author is indebted to Dr. H. A. Charipper for his generous aid in preparation of the photomicrographs.

and contain scattered through them a relatively few but definitely eosinophilic cell types, differing from the typical eosinophiles in their smaller size and in their eccentrically located nuclei. The most constant characteristic, however, lies in the finely granular nature of the cytoplasm, which is almost homogeneous, in contrast to the more coarsely granular cytoplasm of the typical eosinophile cell.

During the fifth day these changes become more pronounced; the vascularity increases, the cell distribution appears even less compact, and the pregnancy-cell type, sparsely scattered in earlier stages, becomes noticeably more abundant.

Sections of daily stages from the sixth to the twenty-second day, the time of parturition, reveal few changes from the condition at the end of the fifth day. Beyond a slow but progressive increase in pregnancy-cell elements with what appears to be a corresponding decrease in eosinophiles until the twelfth day, the gland of these stages remains unchanged (fig. 2).

It seemed of interest to determine what influence the transitory postpartum oestrus, characteristic of the rat, would exercise upon the gland of pregnancy—so far as the pregnancy-cell type is concerned—and whether or not these cells survive this period of heat. Examination of six glands from postpartum rats whose vaginal smears denoted oestrus revealed no change from the gland of pregnancy; the high degree of vascularity was present, and the complement of pregnancy cells was typical of the later stages of gestation.

*The lactation pituitary.* The anterior lobe removed from lactating females at intervals during the suckling period is in no way distinguishable from the gland of pregnancy. Pregnancy cell types persist throughout, and for two days at least after weaning (fig. 3). It was found upon further investigation that premature withdrawal of litters, at parturition and on the fifth, seventh, ninth, and fifteenth days postpartum invariably results in the disappearance of pregnancy-cell types within two or three days, although a certain degree of hyperemia appears to persist for some days longer.

*The pseudopregnant pituitary.* The influence of pseudopregnancy was determined through a study of a series of glands removed at intervals throughout the pseudopregnant period—a period of twelve days in the colony employed. A condition similar to that prevailing during pregnancy was found. The glands give indications of an increasing hyperemia on the second day, in three instances pregnancy cells were discernible on the third day following infertile copulation or cervical stimulation; by the fourth day pregnancy-cell types were quite evident. On the twelfth day, however, these cell types had disappeared, and in some instances none could be found in the eleven-day gland.

Ovaries for each stage were examined and in all cases characterized by the presence of pregnancy cells in the pituitary proved to be predominantly luteal. Coincident with the appearance of regression of the corpora lutea, pregnancy-cell types had disappeared in the hypophysis. The disappearance of pregnancy-cell types coincided in each instance with resumption of the oestrous cycle, as checked by vaginal smears and by examination of ovaries and uteri. Pregnancy cells were not found in pituitaries from animals which were in proestrus or oestrus when killed on the third or fourth day after weaning (fig. 4).

## DISCUSSION

Guerrini ('05) first described the hypophysis in the pregnant dog and rabbit, stating that changes manifest themselves at the beginning of pregnancy, and he ascribed the presumably increased activity to either of two cell types, a granular and a 'plasmosome.' These cell types appeared consistently until parturition, and occasionally could be found for some days longer. These modifications were not discernible in the lactating female, contrary to present findings in the rat. Erdheim and Stumme ('09), working with human material, reported that pregnancy cells made their appearance very quickly after impregnation, persisted throughout gestation and for a considerable time following parturition.

The length of their survival seemed to depend upon the previous reproductive history of the individual, since they were found to persist considerably longer in glands obtained from multiparae. Schenk ('26), in describing the anterior pituitary of the rat during pregnancy, reported that pregnancy-cell types persisted, relatively speaking, a much shorter time than in the human gland. In isolated cases, however, pregnancy cells were detectable as long as six to eight weeks postpartum. Schenk expressed the opinion that undoubtedly many factors, such as number of embryos and previous history, might be influential in causing variations in the duration and condition of the pregnancy pituitary, but no experimental evidence was offered.

Pregnancy changes have been induced experimentally in the non-pregnant animal, notably by Berblinger ('14, '21), Lehmann ('28), Baniecki ('28), Fels ('28), who reported that typical pregnancy changes were induced through administration of foetal and placental extracts. Haterius and Charipper ('31) provoked a pregnancy response in mice through continued implantation of fresh anterior lobe, and attributed the pituitary changes to the ovarian luteinization resulting from the implants. It seems highly probable, indeed, that the response obtained by previous workers resulted from ovarian or ovarian-stimulating principles in the extracts used. Recently, Desclin and Brouha ('31) have reported that in the experimental pseudopregnant guinea-pig typical pregnancy changes manifest themselves, identical with those occurring during pregnancy. Pituitaries recovered from animals, some ten days and some sixty days subsequent to mechanical stimulation, presented a pregnancy picture. Since in one series embryonic influence was excluded, and since in another series hysterectomy removed the uterus as a possible factor, they concluded that the pregnancy changes induced arose from ovarian influence, probably a combination of the follicular and the luteal hormones.

In the present work it is shown that the pituitary of the pseudopregnant rat corresponds with that of the pregnant

animal, in agreement with Desclin and Brouha's observations on the guinea-pig. It is shown further that pregnancy changes occur pari passu with luteinization of the ovaries; modifications do not appear before the corpora lutea of pregnancy are well established. These modifications, however, disappear once the luteal phase passes and cyclic conditions are restored. In view of the close coincidence between luteinization and pregnancy cell types, and the fact that experimental luteinization of ovarian substance results in identical changes, it seems probable that the luteal principle is responsible for these changes.

Results reported here have been obtained with virgin animals, that is, in animals with no previous litter history. How closely these data would apply to the multiparous animal is not known. It is possible, as in the human being (Erdheim and Stumme), that previous reproductive history may result in the persistence of pregnancy changes beyond the time reported in the present work. Just why this should be the case, however, is not clear. Furthermore, the significance of these cell types which appear during the reproductive cycle still remains obscure.

## SUMMARY

In the nulliparous rat, pregnancy changes manifest themselves on the third and fourth day following copulation, both through a marked hyperemia and in the appearance of an eosinophile cell type characterized by a clear, almost homogeneous cytoplasm and an eccentric vesicular nucleus.

These modifications become increasingly marked until the twelfth day of gestation, after which time no pronounced changes are discernible until after parturition.

In the event of lactation, the pregnancy type of pituitary persists until the third or fourth day after weaning, i.e., until just before the oestrous cycle normally should resume. In the event of premature weaning, the same condition obtains.

In the pseudopregnant animal, modifications identical with those occurring during pregnancy are present, persisting throughout the period, and disappearing just before the cycle reestablishes itself.

Ovaries of all stages in which pregnancy changes are discernible are characteristically luteal, and it is believed that the luteal principle is instrumental in the production of the pregnancy cell type.

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## PLATE 1

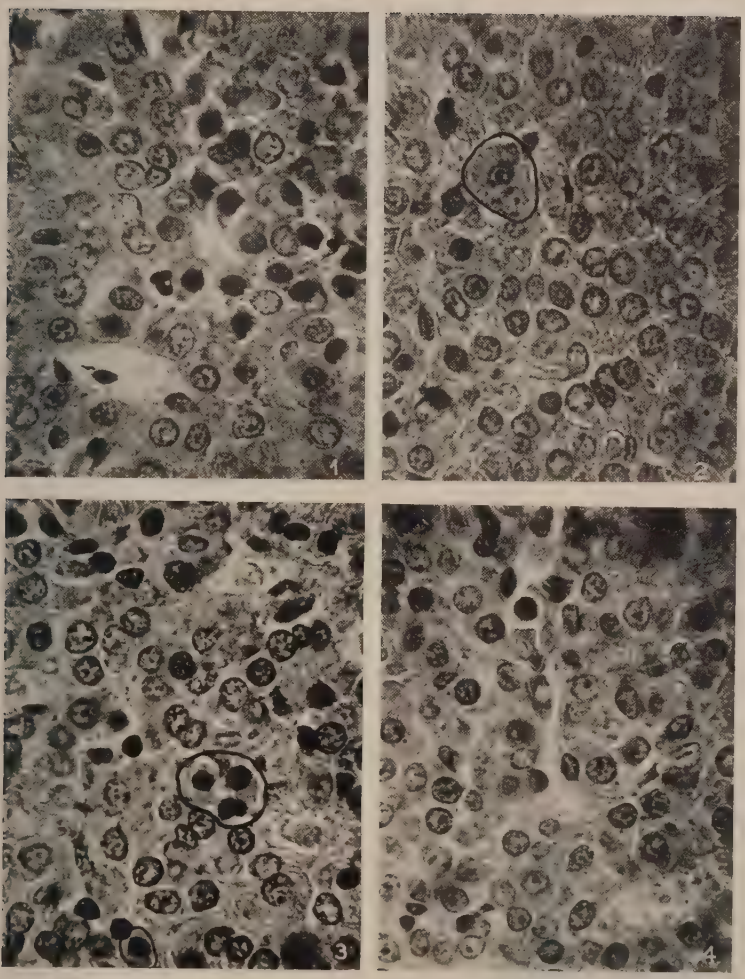
EXPLANATION OF FIGURES

1 Coronal section through the anterior pituitary of rat sacrificed one day after copulation. Groups of epithelial cells, with patent capillaries. No pregnancy cell types. Zenker-formic-osmic: haematoxylin-eosin. × 950.

2 Section through the anterior pituitary of a pregnant rat; removed on the thirteenth day of pregnancy. Pregnancy-cell types encircled. Zenker-formic-osmic: haematoxylin-eosin. × 950.

3 Transverse section through the anterior pituitary removed on the tenth day of lactation. Pregnancy cells encircled. Zenker-formic-osmic: haematoxylin-eosin. × 950.

4 Section through anterior pituitary removed on the fourth day after weaning. Vaginal smear oestrus. No pregnancy-cell types. Zenker-formic-osmic: haematoxylin-eosin. × 950.



# THE STRUCTURE OF THE VAGUS NERVE

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TWO FIGURES

Using the pyridine-silver method, Ranson ('12) found large numbers of sensory unmyelinated fibers in the spinal nerves which he traced to the small cells in the spinal ganglia. He saw the unmyelinated axons of these cells divide in the manner of a Y into a fine branch directed centrally through the dorsal root to the cord and a much thicker branch directed peripherally in the nerve. v. Lenhossék ('07) had previously called attention to the small size of these central branches.

To these findings the objection has been raised by Langley ('22) that the silver methods shrink the nerves so that delicate myelin sheaths are no longer visible and the fine myelinated fibers, being drawn close together by the shrinkage of the connective tissue, resemble bundles of unmyelinated fibers and have been mistaken for them. Kiss and Mihálik ('29) also believed that the silver methods are not sufficient to distinguish the thin myelinated from the unmyelinated fibers. Papers dealing with this objection have recently been published (Ranson and Davenport, '31; and Davenport and Ranson, '31), and we do not need to enter into a discussion of this question here.

In the spinal nerves unmyelinated fibers can be readily demonstrated in good osmic-acid preparations (Langley, '22; and Kiss and Mihálik, '29). In such preparations they have also been seen and very accurately described by Nageotte ('32). A study of the vagus in osmic-acid preparations (Chase and Ranson, '14; Kiss and Mihálik, '31) shows that below the level of the nodose ganglion the myelinated fibers

are rather widely separated by unstained material which in thin sections can be seen to be composed for the most part of unmyelinated fibers with probably no more connective tissue than is present in other cerebrospinal nerves. Most of the large myelinated fibers are given off in the pharyngeal and laryngeal branches. The remaining medium-sized and many fine myelinated fibers leave through the pulmonary plexus. When the nerve enters the abdomen it contains no greater percentage of myelinated fibers than are found in such a sympathetic nerve as the branch from the superior cervical ganglion to the internal carotid artery.

It has been said (Kiss and Mihálik, '31) that there are no unmyelinated fibers in the intracranial rootlets of the vagus in man and the dog and that those found in the cervical trunk and in the thoracic and abdominal portions of the human vagus come from the sympathetic by way of branches of communication between the two nerves. This would make the human vagus as it enters the abdomen practically a sympathetic nerve, and such a microscopical analysis "does not provide an anatomical basis for the existence of a 'parasympathetic' system," Kiss ('31 a). It may be said in this connection that after section of the dog's vagus in the neck, Chase ('16) found degeneration of the nerve as it lies beside the oesophagus in the lower part of the thorax. This led him to believe that the majority of the fibers which it contains are true vagal fibers, although a few additional ones may be derived through anastomoses with the sympathetic.

The object of the present investigation was to see if, putting aside for the moment the silver stains for axons, it would be possible to demonstrate unmyelinated fibers in the intracranial rootlets of the vagus with the osmic-acid stain. It was also proposed to reinvestigate in the cat the anastomotic rami between the vagus and sympathetic in the upper part of the neck.

We have had the opportunity to restudy the preparations of the roots of the dog's vagus described by Chase and Ranson ('14). Two sets of serial sections, one stained with

osmic acid and the other by the Pal-Weigert method, were available. In these series the individual rootlets could be traced to their origin from the medulla. It was easy to see that the rootlets came off in two longitudinal rows, one slightly ventral to the other; and that the rootlets of one row were composed of closely packed myelinated fibers, of which the greater number were small, having, in fact, the size and appearance of preganglionic visceral efferent fibers. These were associated with a moderate number of fairly large fibers of a size suggesting that they might be the ones which, along with others from the bulbar roots of the accessory, supply the striated muscle of the pharynx, larynx, and oesophagus. Very few fibers of intermediate size were seen in these rootlets which, therefore, had much the same composition as the ventral roots of the thoracic spinal nerves. In the rootlets of the other row the myelinated fibers, which were of all sizes, were rather widely separated by unstained material (fig. 1). When this unstained material was carefully examined it was found to be composed of unmyelinated fibers which could be traced in these rootlets up to their point of entrance into the medulla. In cross section the area occupied by rootlets of this type appeared to occupy more than half of the total root area.

We have been able to see the same two types of rootlets in osmic-acid preparations of the cat's vagus, but not as sharply defined as in the dog.

In a cat the left vagus nerve and sympathetic trunk were dissected out without disturbing their relation or the connective-tissue investment that binds them together. They were fixed in formalin containing 5 per cent glacial acetic acid and later stained with osmic acid. Serial transverse sections of these preparations were studied for anastomotic branches connecting the two nerves. Only one connecting branch was found. It connected the vagus just below the nodose ganglion with the sympathetic trunk a short distance below the superior cervical ganglion. It was composed of fine myelinated fibers and probably corresponds to the well-myelinated branch

which Kiss and Mihálik ('30) and Kiss ('31 b) found connecting the two ganglia in man and which he interpreted as the equivalent of a white ramus going from the vagus to the sympathetic. No ramus composed chiefly of unmyelinated fibers was found connecting the two nerves at this level in this series of sections. It is possible that some extremely fine twigs composed of a few unmyelinated fibers may have been overlooked. But it is certain that in the cat the sympathetic does not regularly contribute any considerable number of unmyelinated fibers to the vagus at the level of the nodose ganglion.

Sections of the vagus between the jugular and nodose ganglia and others below the latter show that the myelinated fibers are rather widely separated by unstained material in osmic-acid preparations. When this is examined under high magnification it is possible to see that it is comprised of unmyelinated fibers. The fascicle of the cat's vagus, a photograph of which is reproduced in figure 2, was selected because it contains a relatively small proportion of myelinated fibers. Pains were taken to exaggerate by purely photographic methods the honey-comb appearance of the background. This appearance is due to the presence of closely packed gray rings, surrounding irregular lightly stained areas. These gray rings do not contain a particle of myelin. They are as large, or in some cases even larger, than the smallest myelinated fibers. Nageotte ('32) has interpreted them as representing bundles of unmyelinated axons, each bundle being contained within a single strand of neurilemma.

It seems clear that at least the great majority of the unmyelinated fibers in the upper part of the cat's vagus do not come from the sympathetic. Large numbers of them can be traced through the vagus rootlets to the brain. These fibers must arise from the cells in the jugular and nodose ganglia or from cells in the medulla. Nothing in the observations here reported would exclude the latter possibility.

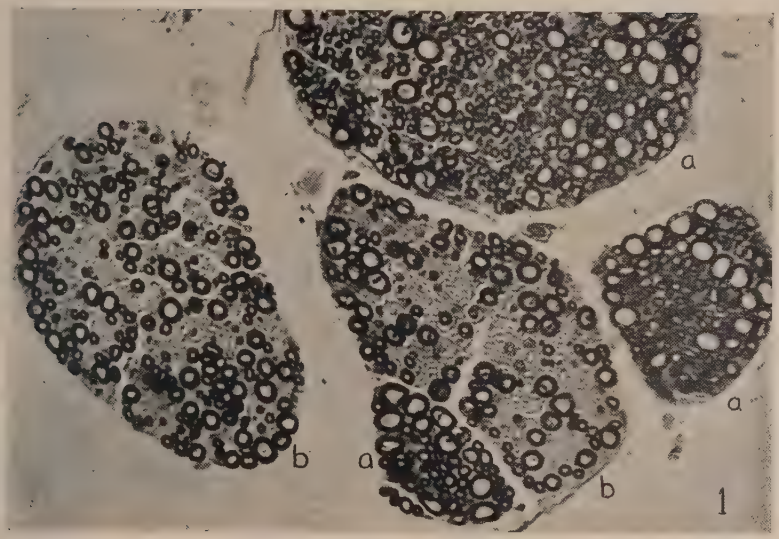

Fig. 1 Two types of rootlets in the vagus of the dog; *a*) probably motor; *b*) probably sensory. Osmic acid. × 400.

Fig. 2 A fascicle of the cat's vagus between the jugular and nodose ganglia selected because of the wealth of unmyelinated fibers which it contains. Osmic acid. × 640.

SUMMARY

In the cat very few unmyelinated fibers are contributed by the sympathetic to the vagus in the region of the nodose ganglion. No well-defined gray ramus was found. A bundle of myelinated fibers was seen joining the vagus, just below the nodose ganglion, with the sympathetic below the superior cervical ganglion.

Two types of vagus rootlets were seen in the dog and cat. One group had a composition similar to that of the ventral roots of the thoracic nerves being composed of closely packed myelinated fibers of two sizes, large and small, with few of intermediate size. The other rootlets were composed of myelinated fibers of all sizes widely separated by great numbers of unmyelinated fibers. The latter can be traced into the medulla and are not of sympathetic origin. Large numbers of these fibers can be seen in the trunk of the vagus above as well as below the nodose ganglion.

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# ACCESSORY BRONCHI IN EMBRYOS; THEIR OCCURRENCE AND PROBABLE FATE

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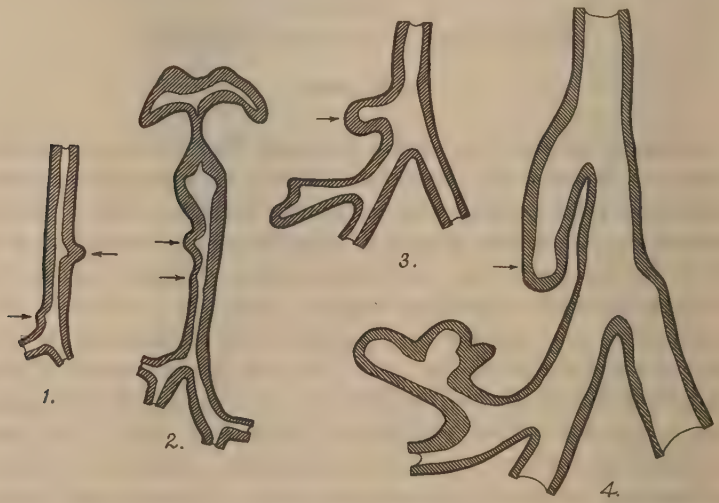
EIGHT FIGURES

A recent acquisition to the Harvard Embryological Collection is a beautifully preserved human embryo of 16.5 mm., sectioned transversely and given the number 2305. On examination there was found a large tracheal bronchus, situated on the right side, arising a short distance above the bifurcation and extending caudally through thirty-five 10 μ sections to end blindly in a rounded tip, with no sign of a bulbous enlargement (fig. 4). Though the presence of this additional bronchus is well known as an anomaly in the human adult, this is the first time, to my knowledge, that it has been noted in the embryo. It seemed desirable, therefore, to make a thorough search in the embryos at my disposal for further instances. Embryos of less than 5 mm. in length were, of course, of no interest in this connection, since the splitting of the trachea from the oesophagus takes place at about that time. Among some eighty human embryos from 5 mm. up to 45 mm., indications of tracheal bronchi were found in three other instances. This, counting the new acquisition, gives an incidence of about 5 per cent, and makes this anomaly worthy of record.

In none of the other cases were the tracheal outgrowths as distinctive as in the 16.5-mm. embryo. They are shown in the reconstructions (figs. 1, 2, and 3) from embryos of 6.25 mm., 8.0 mm., and 10.6 mm., respectively, and can be seen to range from the mere local bulging of the tracheal wall to

well-developed tubular outgrowths, of a caliber comparable with that of the two main bronchi. They vary in position, some being close to the tracheal bifurcation, others higher up, even just below the laryngeal ampulla. Five of the six discovered in this human series are on the right side; only one is on the left. In the two younger embryos (figs. 1 and 2) the outgrowths are two in number, the upper one in each case




Figs. 1, 2, 3, and 4 show the trachea and tracheal bifurcation of human embryos of 6.25 mm., 8.0 mm., 10.6 mm., and 16.5 mm., respectively, in frontal view. Figure 2 shows also the frontal section of the pharynx above and the enlargement which is to form the larynx. Arrows point to the tracheal bronchial outgrowths. Various magnifications.

being the larger and more pronounced. Even the two largest tracheal bronchi (figs. 3 and 4) show no sign of branching, though the main bronchi have already grown branches of the the third or fourth order.

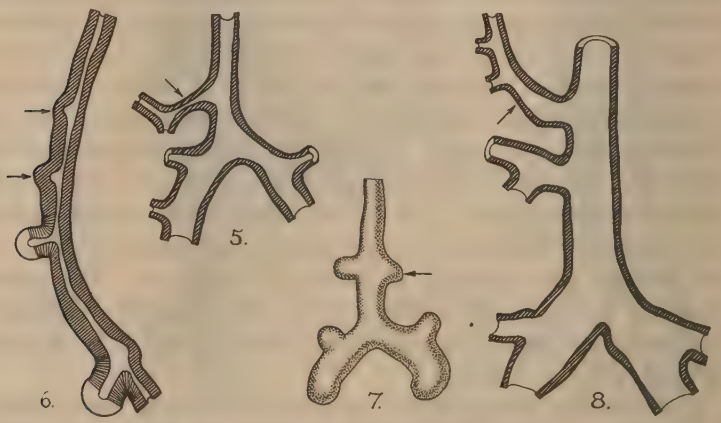
In mammals other than man the presence of a tracheal bronchus on one or both sides is frequently a species characteristic. Lists of those species in which it is normally present are given by Huntington, by Aeby, and by Narath. From the ample material in the Harvard Embryological Col-

lection it seemed wise to study, first, whether tracheal bronchi exist as embryonic anomalies in those species in which they are normally lacking, and, secondly, whether in those species normally provided with the right tracheal bronchus an anomalous extra bronchus might ever occur. The latter possibility is suggested by the conditions shown in the two younger human embryos, each with two extra bronchial buds.

The mammals represented in the collection by a sufficient number of specimens to make such a search at all profitable are man, rabbit, cat, and rat, all normally without the tracheal bronchus; and sheep and pig, in which this bronchus is normally present. Among the cat series of forty specimens of appropriate ages and among the thirty rat specimens, no extra bronchi were found. The rabbit series included seventy embryos, and one instance of the anomalous additional bronchus was detected. This is in a rabbit embryo of fourteen days eighteen hours (circa 12 mm.); the reconstruction of the trachea and bronchi is given in figure 5. The extra bronchus is on the right side near the tracheal bifurcation, is smaller in caliber than the main bronchi, but has already divided into two branches.

In the sheep the anomaly occurs in only one case among the forty-five specimens available. Two small tracheal buds are present above the normal right tracheal bronchus in an embryo of 12 mm. (fig. 6). The lower one is the larger of the two, and both are distinctly dorsal in position, thus differing from all of the other anomalous bronchi found in this investigation. The broad bulging of the ventral wall of the trachea does not indicate the presence of a third anomalous bronchus, but represents in profile the normal enlargement, called by Huntington the tracheal lacuna or bulla, found in this position in many mammals. In the pig two cases of extra bronchi have been discovered. One, noted and modeled by Prof. F. T. Lewis some years ago, was among the 150 embryos of 10 to 12 mm. in length available for class teaching; the other is in an older embryo of 23.6 mm. in the graded series of 110 specimens in the collection. The younger one

(fig. 7) shows the extra bronchus as a well-developed tubular bud arising from the left wall of the trachea nearly opposite the normal right tracheal bronchus, which it closely resembles. The result is that the trachea and the four bronchi make of this model a virtually symmetrical figure, and it was for this reason that Doctor Lewis was especially interested in the specimen. In the older pig embryo, on the contrary, the additional bronchus is on the right side above the normal




Figs. 5, 6, 7, and 8 show the trachea and tracheal bifurcation of embryos of rabbit, fourteen days eighteen hours; sheep, 12 mm.; pig, 10 to 12 mm. (model by Lewis); and pig, 23.6 mm., respectively. Frontal view, except figure 8, which is lateral. Arrows point to supernumerary bronchial outgrowths. Various magnifications.

tracheal bronchus (fig. 8). It is directed obliquely upward and has many branches which form an apical lobe of the lung, already definitely marked off by fissures.

One other group of cases may be mentioned here. In 1897 d'Hardiviller, in a study of the development of the bronchial tree in rabbit embryos, described a bronchial bud situated on the left side in the angle between the trachea and the left stem bronchus. It is figured in six reconstructions from embryos of twelve days twenty hours to fourteen days. In the youngest embryo it lies definitely on the trachea, and

therefore may be considered relevant in the present study. In the older embryos it is shown as a branch of the left stem bronchus. It becomes constricted off from the bronchus, exists for a short while as a thick-walled vesicle, and then vanishes. In spite of its evanescent history, d'Hardiviller considered this left eparterial bronchus as a normal phase of development and hailed it as comparable to that on the right side, thus demonstrating the primitive symmetrical pattern of the mammalian lung. Huntington could not confirm the presence of this bud in rabbit embryos studied by him, nor in embryos of cat and rat, but did find similar relations in one adult rabbit, in a series of seventy rabbit lungs examined, and recalled that Narath reported similar conditions in two individuals. He considers that d'Hardiviller's observations were made on "variant embryos which, if development had proceeded, would have yielded atypical adult individuals possessing the above described left bronchial variant, but that no warrant is given for the assumption that such embryonic variants have a phylogenetic significance in the interpretation of the normal intrapulmonary architectonics." For the present paper the interest in d'Hardiviller's figures lies in the fact that at least one of his rabbit embryos possessed a tracheal bronchial bud situated on the left side just above the tracheal bifurcation; and the failure of Huntington to find similar buds in rabbit, cat, or rat helps, by implication, to swell the total number of those embryos examined for this purpose.

If any value can be attributed to figures of such small magnitude, it seems that this anomaly is more frequent in man than in the other mammals examined. Man shows six tracheal buds in four specimens out of eighty; sheep, two extra buds in one specimen out of forty-five; rabbit, one case out of seventy (+ at least one figured by d'Hardiviller); pig, two cases out of 260; cat and rat, none (including in the number examined those seen by Huntington).

The significance of the supernumerary bronchi in man and of the normal presence of eparterial bronchi in certain mammals has been discussed by Huntington. He considers them as

evidence of the 'migration' of the first dorsal branches of the primary bronchi on to the trachea, and maintains that the primitive lung is of the 'bilateral hyparterial type' found in Hystrix, to which the eparterial branches of the various lung forms have been added by the separation of existing components and alteration of their original relations to the bronchial stem and the pulmonary artery. This view is accepted by Schneider, who insists that these bronchi should not be considered as accessory in the sense of new formations, even though the first dorsal branch of the primary bronchus coexists with a tracheal bronchus on the same side. Others, led by Aeby, think the primitive type is represented by lungs with bilateral eparterial bronchi, as in Equus, Phoca, etc., and that all other existing lung types have been derived from this type by the loss of certain bronchi or bronchial branches. For these authors the additional bronchi would be due to the abnormal retention of ancestral characters. The question seems to me to be largely academic, and cannot be settled by analysis of the few examples of extra tracheal buds or bronchi shown here. The migration theory is apparently denied by the presence of two supernumerary buds on the same side and by their high position in one instance (fig. 2). It is true that the youngest human embryo (fig. 1) and the younger pig embryo (fig. 7) might well be considered as examples of the return to an original symmetrical lung pattern presenting bilateral eparterial bronchi; but the reduction theory does not fit the facts either in the case of the second human embryo (fig. 2) or in that of the older pig embryo (fig. 8), in both of which there are two tracheal bronchi on the right side, for both these cases apparently show redundancy instead of reduction.

The fate of these anomalous bronchial buds may apparently vary in different instances. Judging by the relatively high percentage found in the human embryos as compared with the known rarity of the condition in the human adult, we must suppose that many of them disappear without leaving any trace. This may be by simple absorption or by the formation

of pediculated vesicles, as described by d'Hardiviller, though I have seen no sign of such a vesicle in my specimens. It has been suggested that their former presence may account for certain irregularities in the tracheal cartilages or for weak areas in the musculature. The surviving buds may form tracheal diverticula, accessory lobes of the lung, or probably one type of tracheocele.

Whether the bud is to form a simple diverticulum or a lobe of the lung apparently depends on its position, the direction of its growth, and its growth power. Pathologists (e.g., Henke and Lubarsch, '28) recognize a type of 'traction diverticulum.' This is situated on the dorsal wall of the trachea and extends toward the oesophagus. In other instances 'traction diverticula' of the oesophagus occur. Both may be considered as evidences of the irregular separation of the tracheal keel during embryonic development, which may lead to the well-recognized anomalous connection between trachea (or bronchus) and oesophagus usually accompanied by atresia of the oesophagus at a higher level (Lewis). These diverticula are not, therefore, outgrowths from the tracheal wall, but remains of the former undivided cavity, pulled out, as it were, by the separation of the two tubes. Sometimes they are connected with the other tube by strands of connective tissue. They are passive structures and do not grow in the sense of sending out branches, though they may become quite large sacs. In the present series they are represented by the two buds from the middorsal wall of the trachea in the sheep (fig. 6).

Each of the two oldest human embryos shows a single tubular tracheal outgrowth from the right side. In one (fig. 3) the tube grows laterally, and the mesodermal mass into which it projects already protrudes into the pleural cavity. The tube has not yet branched; it lags in this respect somewhat behind the right bronchus, which is already giving off branches of the second order, but this is only to be expected when one considers the late origin of the tracheal buds as shown in the younger human embryos. Comparison of this

laterally directed tracheal branch with those of the somewhat older rabbit embryo and the still older pig embryo (figs. 5 and 8) leads us confidently to expect that in this human embryo the branch would have later developed branches and ultimately lung tissue, and that this would have either been a part of the usual apical lobe or formed a separate lobe, above the latter. Bàrsony describes such a lobe as the "apico-dorsal lobe of the lung, a heretofore unknown localization of the azygos lobe." (The lobe commonly called azygos is a representative of the cardiac division.) Another such lobe is described by Carnière and Huriez as the "apical lobe of the azygos vein." Those cases in which the lung tissue fuses with that of the true apical lobe form the group of anomalies in which the presence of the tracheal bronchus is the main feature. The level of the bronchus on the trachea may well decide whether it is to grow into the common ala pulmonalis or form a separate bulge at a higher level, but still in the region of the pleural coelom, leading to the formation of a separate lobe with intervening fissures.

The tracheal bronchus of the oldest human embryo (fig. 8) points caudally. It still shows no sign of branching, though the normal bronchi bear branches of the third or fourth order. By comparison with the rabbit embryo at least primary branches might be expected. Furthermore, it is growing less rapidly, judging from the count of mitotic figures in its tip, than the end branches of other parts of the same bronchial tree. Its direction of growth is not toward the pleural coelom, but into the mesenchymal mass destined to become the mediastinum.

This tracheal bronchus was destined, in all probability, to become a true tracheal diverticulum. As described by Chiari, these, in the adult, are tubular without bulbous enlargement, several centimeters in length, the wall resembling that of the trachea in epithelium and usually in the presence of cartilaginous rings and musculature. Schneider reports also analogous diverticula from the main bronchi. They represent branches of the bronchial tree which for some reason have

stopped short in their development. It is possible, however, that the embryonic bronchus in question might have continued its growth to form a rudimentary lung, even in spite of the dense tissue in which it would have been forced to develop. Schneider notes one case in which a compressed mass of atelectatic lung tissue was found in the posterior mediastinum, and Stibbe describes what probably should be considered an abortive lung behind the manubrium sterni.

In Stibbe's case the mass or 'tumor' was 'a large round cyst-like body,' lying free in the loose tissues at the root of the neck behind the manubrium, and probably not palpable during life. It was connected by a long pedicle with the back of the trachea, about an inch above the bifurcation. The pedicle allowed the passage of a fine probe and opened by a pin-point opening with rounded edges. The mass consisted of a number of irregular, rounded spaces lined with the two-layered, ciliated, columnar epithelium characteristic of the respiratory tract. Muscular tissue was not observed and cartilage was absent.

As I read the developmental history of this case, it started with the outgrowth of such a tracheal bronchus as is seen in the largest human embryo here figured. Growth continued to the formation of several bronchial branches and bronchioles, but this embryonic lung could not expand properly in the dense tissue median to the pleural coelom, and never put out the final branches which would form the infundibula and alveoli, with their characteristic epithelium. During the development of the neck and the relative descent of the thoracic organs, the expanded end of the bronchus remained in its original relation to the thyroid gland, passing to the front of the trachea as it found more room. There may even have been an attempt to form a pleural sac around it, since it was said to have been 'shelled' out without dissection. Since only the duct system of the lung was represented, the appropriate epithelium alone was present; the failure of the bronchial cartilage and musculature to develop may be correlated with lack of function, and is sometimes seen even in the simple

tubular diverticula. Stibbe considered this case to be one of congenital tracheal diverticulum, a rudimentary third-stem bronchus, but thought that it had failed 'to join the lung,' not recognizing the tumor-like mass as a rudimentary accessory lung.

The third possibility as to the fate of the tracheal buds is that they may ultimately form tracheoceles. This condition is a well-recognized abnormality and consists of the presence of a sac usually of large size when first noticed situated in the root of the neck and containing sometimes a certain amount of fluid, but also, and distinctively, air, which can be increased in amount by the patient on closure of the larynx, and expressed into the trachea by pressure on the surface. These 'tumors,' originally mistaken for the penetration of air into the thyroid gland and therefore termed aerial goiters, have also been called bronchoceles, fistulas, or hernias of the trachea, gaseous tumors of the neck, and aeroceles, and are related to the laryngoceles, or abnormal extrusions of the lateral pouches of the larynx similar to the 'roaring sacs' of the howling monkeys, from which they are differentiated by the attachment of their hollow pedicle to the trachea. They are to be distinguished from cases of the pathological erosion through the wall of the trachea and the consequent escape of air into the tissue spaces of the neck.

Among others, Petit has described many cases of tracheocele in a long thesis. He quoted an English surgeon, stationed in Egypt, as reporting in 1820 that the condition was common among the muezzins who call the prayers of the Koran from the minarettes, and had also been seen in two English drill sergeants. Obviously, the forceful use of the voice may be a contributing cause. In other cases the tumor was first noticed after violent fits of coughing or of vomiting, after great physical exertion in laborers, or after child birth in women, but many cases are congenital. The sac is usually described as thin-walled, and in some of the few cases where histological examination has been attempted the lining epithelium was found to be composed of ciliated cells.

Earlier authors were inclined to regard this rare anomaly as a congenital opening of the trachea into the thyroid gland or as the remains of some of the branchial pouches. With better understanding of embryological development and the knowledge that the thyroid develops by an outgrowth from the root of the tongue (thyreoglossal duct) and that the branchial pouches are related to the lateral borders of the pharynx, and not to the median keel which is to form the trachea, these causes have been discarded, and at present the condition is considered as either the abnormal enlargement of one of the tracheal glands or as the actual hernia of the trachea through a 'weak spot' in its wall. It is pointed out that the bodies of the tracheal glands lie outside the trachealis muscle, and therefore are not protected by it from abnormal expansion. It is possible that some cases may be traced to this origin. Many pathologists consider the hypertrophy of the mucous glands responsible for the cases of multiple small hernias of the trachea, and others think this possible only as a sequel to chronic catarrh. But the presence of ciliated epithelium in some of the sacs makes this origin doubtful in these cases, for in an inflated gland one would expect glandular epithelium without cilia.

The simple hernia of the tracheal wall should produce a sac with ciliated lining. Against this interpretation of the result is the fact so frequently emphasized in the literature of the sudden appearance of large tumors. One would expect the 'weak spots' in the wall to expand more slowly. On the other hand, if these were already present as tracheal diverticula, perhaps grown to a considerable size, with a narrow inlet closed to the ordinary intratracheal pressure, but forced by an excessive pressure, one can imagine such a rapid expansion resulting in a noticeable tracheocele. If the diverticulum had remained unbranched, a simple cyst would result; if the normal bronchial branching had occurred, the tumor would be lobulated. One can imagine that, in Stibbe's case described above, the forcing of air through the narrow, hollow pedicle would have resulted in a typical tracheocele, expanded rapidly

and therefore noticeable for the first time, lobulated, and lined with ciliated epithelium.

The constant relation of the hollow pedicle to the tracheal wall is emphasized in descriptions of cases of tracheocele. In almost all, this union takes place along the line of the ends of the cartilaginous rings, where they meet the tracheal muscle, and in the great majority of cases on the right side. In one or two cases a diverticulum issues between two tracheal rings. Huntington has discussed the dextral prevalence in the normal tracheal bronchi of different classes of animals, and accounts for it by the asymmetrical disposition of the thoracic viscera and the vagus nerves in the embryo. In the present series only two of the tracheal diverticula are on the left. The commonly dorsolateral position of the abnormal bronchi and pedicles of tracheoceles has not been explained. In the embryo the outgrowths are usually frankly lateral. It seems possible that the change is to be accounted for by an unequal growth in the circumference of the tracheal wall. The centers of cartilage formation are known to be located in the midventral wall, and to spread laterally in both directions. There may be an actual increase in the substance of the ventral wall, carrying the former lateral material to a more dorsal position, aided perhaps by the sinistral spiral twist of the trachea noticeable in many embryos. On the other hand the larger tracheal bronchi normally present in certain mammals, as figured by Aeby and by Narath, seem to proceed from the lateral wall, the tracheal cartilages arranging themselves irregularly around them. The cause of the prevalence of the cartilage-muscle junction as the site of the smaller pedicles of the tracheoceles must therefore be considered as uncertain.

Frequently in the literature concerning tracheal diverticula and accessory bronchi, one encounters the statement that the condition is much more prevalent in the male than in the female. Thus, according to Henke and Lubarsch, cases of tracheal diverticulum are always on the right side and notably always in the male, with the exception of one instance known

to them. Chiari reports five cases all in males; Schneider also mentions this practical limitation as to sex. On the other hand, there seems to be no inherent cause for such a limitation in the development of extra tracheal branches. Certainly, in those animals in which the tracheal bronchus is the rule the condition exists in both sexes, and in the literature of tracheoceles, which I have here considered as a further development of tracheal diverticula or accessory lungs, the prevalence of the condition in males ceases to be stressed. The strains of child birth are frequently mentioned as a contributory cause; Stibbe's case was found in a woman, and congenital tracheocele has been reported more than once as occurring in female infants. Petit gives his observations on thirty-two cases of spontaneous aerocele, of which nineteen were male and thirteen female. In the present series of embryos showing diverticula, only two are old enough to determine the sex, but of these one is female, the other male. I am inclined to think that the supposed practical limitation of these conditions to the male sex is based on fictitious premises. It is probable that when the development leads to a large 'tumor' which can be readily recognized in the living subject, the condition is found equally in both sexes, but that conditions discernible only after death (except by the most modern methods of bronchoscopy) would be divulged chiefly in the dissecting room, where the population is notably predominantly male.

## SUMMARY

Tracheal buds or bronchi have been found in four human embryos and in one rabbit embryo. In two cases two buds are present at different levels on the same trachea. Among those animals in which the right tracheal bronchus is a normal constituent of the bronchial tree, additional bronchi have been demonstrated in one sheep embryo and two pig embryos, which seems to imply that they are the expression of redundancy, and not of a return to a primitively symmetrical system characterized by bilateral eparterial bronchi.

In man, the large percentage of their occurrence in the embryo as compared with the number of reported cases in the adult makes it probable that many of them are resorbed into the tracheal wall. Those surviving may become bronchial diverticula, accessory bronchi leading to the normal apical lobe, to an accessory apical lobe, or a rudimentary third lung in the mediastinum. The result in the individual cases seems to depend on the position of the tracheal bud, and the direction and duration of its growth. Either the bronchi without branches and with bulbous terminal enlargements, or those with a certain amount of embryonic lung development may be converted, if the lumen of the stalk is still patent throughout, into the congenital form of tracheocele or that form called spontaneous which appears after sudden, violent intratracheal pressure.

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# THE HISTOGENESIS OF THE CLAVICLE IN THE ALBINO RAT [1]

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TWO PLATES (TWELVE FIGURES)

The development of the human clavicle has been a subject of much controversy since the middle of the last century. During this period numerous workers have presented various theories of clavicular origin. Gegenbauer ('64) was the first to recognize that the developing clavicle presented an unusual pattern, and recently Hanson ('20) stated that the last word concerning the origin of this element of the human skeleton had not yet been spoken.

A review of the literature on the formation of the clavicle reveals three outstanding theories. According to the first, the clavicle is entirely preformed in cartilage and is therefore a part of the primordial skeleton. A second theory, supported by numerous investigators at different times, attributes to it a purely intramembranous origin. The third, also supported by a large group of anatomists, describes the clavicle as forming from both cartilaginous and intramembranous elements.

Gotte ('77) and Hoffmann ('79) considered the human clavicle to be a cartilage bone. Broom ('99), Fawcett ('13), and Hanson ('20) maintained that the cartilage present had no morphological significance and that therefore the clavicle was as much a pure membrane bone as the mandible. Paterson ('02), Fitzwilliams ('10), and Huntington ('18) believed that it arose from more than one morphological unit; that

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is to say, that its acromial end ossified directly in membrane and the sternal end ossified indirectly through replacement of cartilage.

This confusion of ideas has found its way into well-known texts on anatomy and embryology. Foster and Balfour ('93) and Morris ('07) tended to accept the theory of the dual origin of the clavicle. Wilder ('23) avoided making an interpretation regarding the origin of the mammalian clavicle by stating that:

> One is not always certain if the bone that is called the clavicle is really this dermal element alone, or a fusion of the two elements, the presence of cartilage cells in the embryo being taken as an indication of the presence of both a clavicular and a procoracoidal element.

Statements, equally non-committal, were made by Gray ('01), McMurrich ('02), Piersol ('18), Prentiss and Arey ('20), and Cunningham ('26).

This divergence of opinion may be due in part to the failure of any one investigator to make careful histological study of the developing clavicle in an accurately timed series of embryos. It has, therefore, seemed significant to study the histogenesis of the clavicle in an experimental mammal, the albino rat, from which carefully timed embryos can be readily obtained.

## MATERIAL AND METHODS

A breeding colony from The Wistar Institute albino rat stock was raised. The animals were kept on an adequate diet. The oestrous cycle of the breeding females was followed by the use of the vaginal-smear technique described by Long and Evans ('22). The female was mated during the period of oestrum and a successful mating was always determined by microscopic observation of spermatozoa in the vaginal smear or by the presence of the vaginal plug. The age of the embryos was reckoned from the time of the fertile mating.

Ovulation in the rat occurs spontaneously, and, unlike the case in the rabbit, is independent of copulation. The most accurate attempt to time rat embryos is, therefore, open to a pos-

sible variation of twelve hours, for, as Long and Evans ('22) have shown, ovulation may occur from eighteen to thirty hours following the onset of oestrum.

This report is based upon the microscopic study of serial sections of the clavicle from eight rats in which the ages were accurately determined within the limits defined above. The youngest stage observed was fourteen days and eight hours intra-uterine. The oldest stage studied was seven days post-uterine.

The material was fixed in Bouin's picro-formal fluid and when decalcification proved necessary the phloroglucin and nitric-acid technique was used. Maximow's haematoxylin, eosin-azur stain was found to be the most satisfactory in that it clearly differentiated protochondrium, cartilage, calcified cartilage, and bone.

## HISTOLOGY OF THE CLAVICLE

### *Stages: Fourteen day eight hour and fifteen day eight hour; intra-uterine*

No evidence of clavicle formation, except a very slight thickening of mesenchyme, could be found in the fourteen day eight hour embryo. Twenty-four hours later, however, a dense concentration of mesenchyme appeared in the clavicular region, and within this mass the first signs of bone formation were apparent. With Mallory's connective tissue stain this osseous material appeared as blue strands among red-staining mesenchymal cells (figs. 1 and 8). These strands of primary bone formed a network between the connective tissue cells, among which it was as yet impossible to distinguish osteoblasts. This pattern so completely coincides with Maximow's ('31) description of the first stages of the formation of intramembranous bone that it leaves no doubt in the minds of the authors that the primary ossification center in the rat clavicle is of intramembranous origin.

*Stage: Sixteen day eight hour; intra-uterine*

The clavicle of the sixteen-day embryo had increased in width and thickness and had grown in length in the direction of the sternum. A dense mass of mesenchyme still appeared as the clavicle anlage. A marked increase in the amount of membrane bone, confined to the acromial region of the clavicle was apparent (fig. 2). No mature bone cells could be found embedded within the osseous material, which fact was taken to indicate that the process of ossification was still in the early stages of development. Osteoblasts, however, could now be distinguished, arranged in rows along strands of bone. At the sternal region of the clavicle, in close proximity to the membrane bone, appeared a large, oval mass of protochondrium. This was the first foreshadowing of cartilage development.

*Stage: Seventeen day eight hour; intra-uterine*

A marked increase in the amount of membrane bone at the acromial end appeared in the seventeen-day stage. The protochondral mass at the sternal region had become differentiated into typical hyaline cartilage (figs. 3 and 9). This cartilage shaft was encased, except for its sternal extremity, by a thin layer of newly formed bone. Inasmuch as this bony material covered a true cartilage shaft which later became replaced by endochondral bone, it appears to the authors only logical to define this outer covering as true perichondral bone.

Figure 10, a magnified portion from an adjacent section of the same series as figure 9, shows the first histological suggestion of endochondral ossification. The cartilage cells appeared vesicular and the hyaline matrix showed the characteristic calcification which occurs just prior to the inward migration of osteogenic tissue destined to form endochondral bone.

*Stage: Eighteen day eight hour; intra-uterine*

Figure 4 shows diagrammatically the process of development in the clavicle in the eighteen-day embryo. Numerous spicules of endochondral bone appeared within the developing marrow cavity, which was filled with osteogenic and primary marrow tissues. At the acromial end there appeared for the first time a mass of protochondral tissue (fig. 11). This mass was histologically identical with that which appeared at the sternal end on the sixteenth day. Between this protochondral tissue at the acromial end and the spicules of endochondral bone within the marrow cavity, irregular bands of deep-staining bone of intramembranous origin could still be distinguished.

*Stage: Twenty day eight hour; intra-uterine*

The clavicle of the twenty-day embryo showed significant developmental changes which are diagrammatically represented in figure 5. Increase in length had occurred by growth of the cartilage at the sternal end with the typical advancement of perichondral and endochondral bone formation upon this growing tip. At the acromial end the protochondral mass had become differentiated into hyaline cartilage which likewise showed the early stages of replacement by perichondral and endochondral ossification.

At this stage it was impossible to distinguish that portion of the osseous material which was of intramembranous origin, since it had become indistinguishably fused with replacement bone. Figures 5, 6, and 7, therefore, do not include that relatively small portion of bone which first arose directly in membrane.

*Stage: At birth*

No new process in the formation of the rat clavicle at birth could be observed. The growth patterns which had become established in the twenty-day stage, namely, the growth of cartilage at both extremities and the replacement

of both of these by bone, were still progressing at a rapid rate.

Mention might also be made (fig. 6) of the first appearance at this stage of a dense, distinctly separate mass of mesenchyme at the sternal end, within the center of which appeared a core of precartilage tissue. This is undoubtedly the separate element which later ossifies and which has been variously named and interpreted (Dawson, '25; Todd, '28).

*Stage: Seven days; post-uterine*

An examination of figure 7 will show that the clavicle of the seven-day rat has increased in length and width. The bone had elongated by growth at both extremities and there appeared a corresponding increase in the amount of perichondral and endochondral ossification. A large marrow cavity filled with osteogenic and primary marrow tissues appeared. A study of the photograph in figure 12 will reveal many histological details typical of the formation of endochondral bone. A narrow band of perichondral bone appears at the base of the photograph and the marrow cavity is filled with typical connective tissue elements. Spicules of calcified cartilage appear at the border of the rapidly changing cartilage shaft. Along the edges of these spicules, which photograph black, there may be seen deposits of endochondral bone, which photograph as grayish borders.

## DISCUSSION

From the foregoing description, it is evident that the clavicle of the rat formed as a bone of dual origin. The first hint of ossification in this skeletal element appeared in the fifteen-day embryo as intramembranous, bony strands. On the seventeenth day, true hyaline cartilage and replacement bone first appeared at the sternal end, and on the twentieth day similar tissues were identified at the acromial end. It was evident that the clavicle of the twenty-day embryo was developing by a process of fusion of the membrane bone with the cartilage bone of both extremities. The same processes were

rapidly progressing in the developing clavicle in the young rat seven days following birth.

Recent controversies concerning the development of the clavicle have been due primarily to the interpretation assigned to the cartilaginous tissue involved in the process. If investigators considered this tissue histologically typical of true cartilage, some were inclined to conclude that it held no morphological significance in the ossification process. Such was the conclusion of the most recent report, that of Hanson ('20), who, because of this view, considered the human clavicle to be of purely intramembranous origin.

The description of the development of the clavicle given in this report showed that the histogenesis of the cartilage tissue followed typical patterns. Protochondral tissue differentiated out of a concentrated mesenchymal mass. This protochondral tissue soon showed the microscopic anatomy characteristic of true hyaline cartilage. Indeed, the histogenesis of the cartilage in the rat clavicle followed so closely the descriptions recently given by Maximow that there appeared no evidence upon which to classify any tissue at any stage in the process as 'peculiar' or 'indifferent.'

The cartilage shafts which appeared at the sternal and acromial ends in the rat clavicle possessed the utmost morphological significance, for, upon their foundation, replacement bone was formed. Furthermore, the details of the replacement patterns appeared typical in every respect. Perichondral ossification preceded slightly in advance of the formation of endochondral bone. The numerous, well-known histological changes which occur in the development of endochondral bone were all identified. In fact, although the development of the clavicle first began with the appearance of membrane bone, its final modeling, especially its growth at both extremities, was dependent principally upon the formation of replacement bone.

The study of the histogenesis of the rat clavicle confirms in every respect Huntington's ('18) statement regarding the evolution of this mammalian element. No better summary

can be given than that expressed by him when he outlined the recapitulation which the formation of the mammalian clavicle illustrated. Huntington stated that:

> . . . in the unique ontogeny of the mammalian clavicle, the primary direct center of the ossification of the shaft, appearing without preceding formation of cartilage, . . . . represents the dermal element of the bone, derived from the exoskeleton. The cartilage added to this primary center at both the sternal and scapular ends is derived from the cranial element of the primitive coracoid, the procoracoid, and forms the bed in which further ossification of the shaft of the bone proceeds in both directions.

## SUMMARY AND CONCLUSIONS

1. Histological studies of the clavicle have been made from a series of carefully timed embryos of the albino rat.

2. The first hint of ossification appears as intramembranous bone in the fifteen-day embryo. Twenty-four hours later, a mass of protochondral tissue develops at the sternal end. On the following day this becomes differentiated into true hyaline cartilage around which perichondral bone is forming. Similar growth patterns appear at the acromial end on the eighteenth and twentieth days. The first endochondral ossification appears on the eighteenth day at the sternal end. By the twentieth day, this same process can be observed at the acromial end.

3. The clavicle of the rat develops as a bone of dual origin. The first osseous material to appear is membrane bone, which increases in amount, and within three days becomes indistinguishably fused, first, with cartilage bone forming at the sternal region and then with replacement bone developing at the acromial area. The clavicle of the rat seven days after birth shows growing cartilage at both extremities, and it is clear that its development at this stage is entirely dependent upon periosteal and endochondral ossification.

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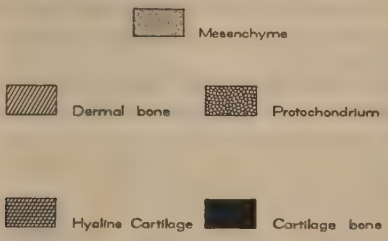
## PLATE 1

### EXPLANATION OF FIGURES

Figures 1 to 7 give a diagrammatic scheme illustrating the ossification process in the rat clavicle.




1 Fifteen day eight hour rat clavicle. × 29.
2 Sixteen day eight hour rat clavicle. × 29.
3 Seventeen day eight hour rat clavicle. × 29.
4 Eighteen day eight hour rat clavicle. × 29.
5 Twenty day eight hour rat clavicle. × 29.
6 The clavicle in the newly born rat. × 21.5.
7 The clavicle in the week-old rat. × 21.5.
8 Photomicrograph showing the region of ossification in the fifteen day eight hour rat clavicle. × 600. (Compare fig. 1.)

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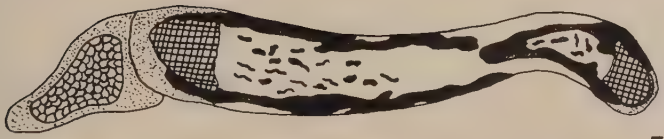

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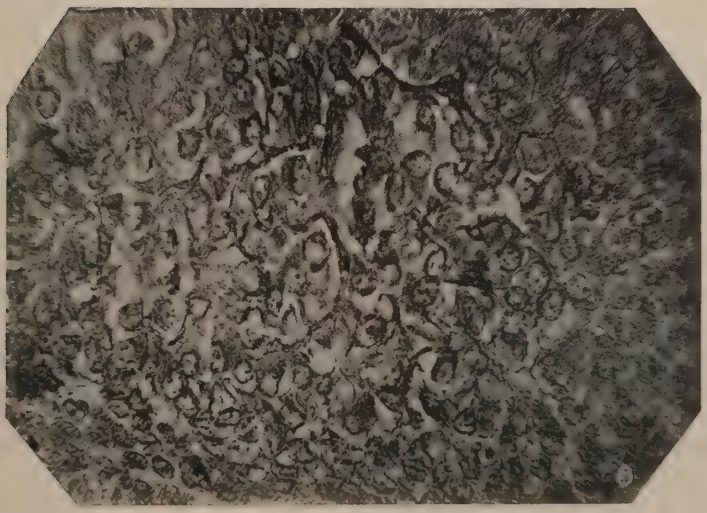


## PLATE 2

### EXPLANATION OF FIGURES

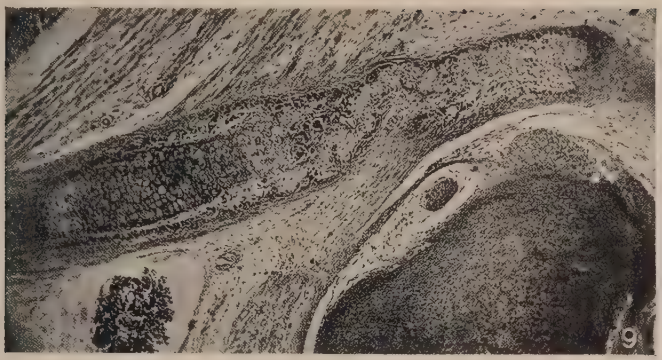
Figures 10 to 12 are photomicrographs of significant stages in the process of ossification in the rat clavicle. The sternal end of the clavicle is at the left in each figure.

9 The seventeen day eight hour rat clavicle, showing the cartilaginous mass at the sternal end. Perichondral bone is just beginning to form around this cartilage. Intramembranous ossification appears in the acromial region. × 67. (Compare fig. 3.)

10 A more highly magnified portion of the lateral end of the cartilaginous mass illustrated in figure 9, showing the first changes of the cartilage mass just prior to the invasion of the osteogenic tissue destined to form endochondral bone. × 200.

11 The eighteen day eight hour rat clavicle, showing the first appearance of protochondral tissue at the acromial extremity. × 100. (Compare fig. 4.)

12 Endochondral ossification at the sternal extremity of the clavicle of the week-old rat. × 67. (Compare fig. 7.)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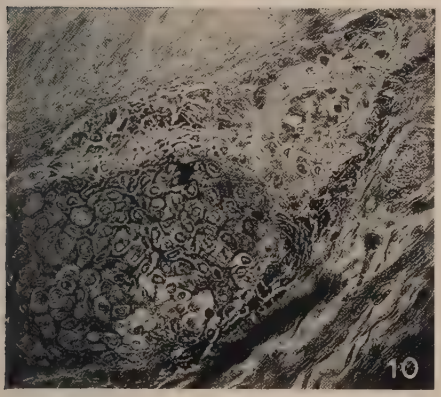
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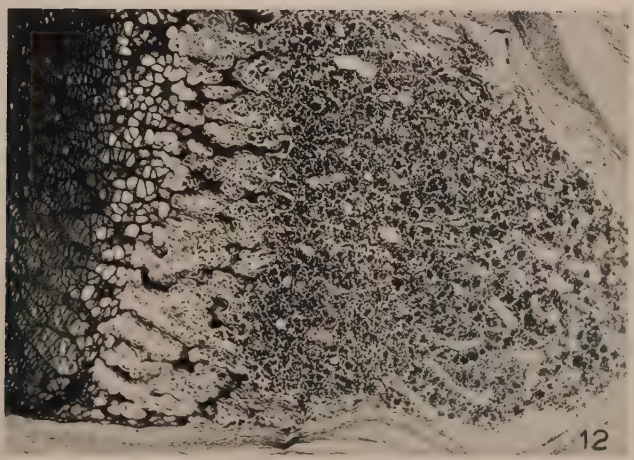
12

# INNERVATION OF THE LARYNX

## II. RAMUS ANASTOMOTICUS AND GANGLION CELLS OF THE SUPERIOR LARYNGEAL NERVE[1]

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FIVE TEXT FIGURES AND TWO PLATES (TWELVE FIGURES)

The ramus anastomoticus might be considered as the terminal twig of the internal ramus of the superior laryngeal nerve. It passes downward under cover of the thyroid cartilage in the posterior part of the larynx to join the recurrent laryngeal nerve at the latter's entrance into the larynx. Although it represents a possible pathway for exchange of fibers between the superior and recurrent laryngeal nerves, its exact character and distribution have never been determined. The ramus anastomoticus therefore represents an unknown factor in the problem of laryngeal innervation.

Ganglion cells have been described by various investigators along the course of the superior laryngeal nerve. These cells are of special interest in that they may be associated with the problem of sympathetic innervation of striated musculature. There has been some suggestion in the literature that tonic and trophic impulses reach the laryngeal muscles through the superior laryngeal nerve, while voluntary motor impulses pass by way of the recurrent laryngeal nerve (Exner and Pineless, '91).

[1] Read in part before the Chicago meeting of the American Association of Anatomists, April 3, 1931. Through the kind generosity of Dr. S. W. Ranson, part of this work was performed in the laboratories of the Institute of Neurology, Northwestern University. Dr. W. A. Willard has rendered special assistance in organizing and interpreting the results. To these two men I wish to express my sincere gratitude and appreciation.

## MATERIAL AND METHODS

The complete anatomical pattern of the superior and recurrent laryngeal nerves was worked out in man and dogs. Binocular dissection of two human and ten dog cadavers was supplemented by gross dissections in ten human cadavers and graphic reconstruction from serial sections in four pups. For the last the desired region of the neck was dissected and treated en block by Ranson's pyridine-silver method for nerve axons, sectioned, and examined under the microscope.

The function of nerves was then studied by the electrical-stimulation method, using one dry cell, bipolar electrodes, and a Du Bois inductor. The secondary coil was drawn clear out and turned vertical to eliminate 'spread.' Ether anesthesia was used for the motor responses. Decerebrate anemia, after Pollock and Davis ('23), plus amytol and ephedrin, was used in eliciting sensory reflexes.

The normal histology of the various nerves was established in the dog from osmic and silver preparations. The myelin sheaths were stained with 2 per cent osmic for two days. The axones were stained by the pyridine-silver method after drawing through spinal cord to insure staining of the small black unmyelinated fibers (Ranson, '18).

Degeneration experiments were carried out on six adult dogs. The usual surgical aseptic technic was used and the desired nerves resected for 1 cm., to prevent regeneration. The dogs were then killed after complete degeneration was believed to have taken place, about thirty days, and the nerves removed, stained as described under histology, sectioned, and compared under the microscope with the normal nerves.

## ANATOMICAL PATTERN

The ramus anastomoticus exhibits a wide variety of patterns in both man and dog. In its simplest form it passes as a single nerve over the posterior crico-arytenoid muscle to join the recurrent at the latter's entrance into the larynx (fig. 1, *A*). In its most complex pattern it forms an intricate plexus with the terminal branches of the recurrent nerve

(fig. 1, *B*). In these cases part of the ramus anastomoticus may pass through the substance of the posterior cricoarytenoid musce. This is of special significance in relation to the ganglion cells which have been described as being present in this muscle. Twigs to the overlying pharyngeal and esophageal mucosa are given off the ramus anastomoticus in its passage downward to join the recurrent nerve.

In the dog, the further course of the ramus is simplified by the fact that it separates again almost immediately after join-

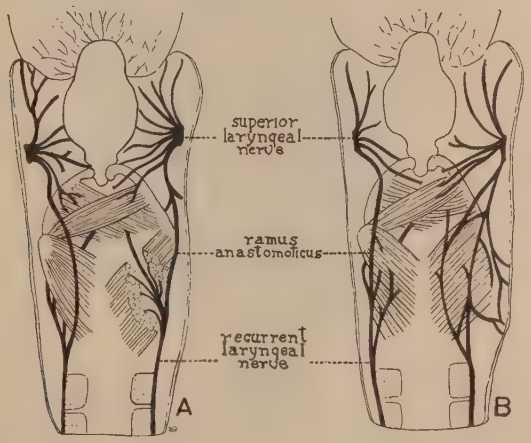


**Fig. 1** Pattern of ramus anastomoticus in man. *A*, simple form. *B*, complex plexus formation.

ing the recurrent to form the upper part of what will be termed the pararecurrent laryngeal nerve (fig. 2). This nerve has been variously described by Onodi ('02), Baum and Ellenberger ('91), Bradley ('27), and a few others. The pararecurrent springs below from the vagus or recurrent near the latter's origin and passes upward in the tracheal-esophageal groove to interdigitate with fibers coming from the ramus anastomoticus above. In the dog the pararecurrent innervates the trachea and esophagus, while the recurrent contains only fibers going to the laryngeal muscles. The pararecurrent

usually runs as a single trunk or as a plexus formed by several parallel trunks. Occasionally, in the dog, as is always true in man, the pararecurrent remains incorporated in the recurrent proper which then innervates the trachea and esophagus as well as the laryngeal muscles.

It is impossible to determine by purely anatomical methods to what an extent the pararecurrent is composed of fibers from the ramus anastomoticus above or from the vagus or

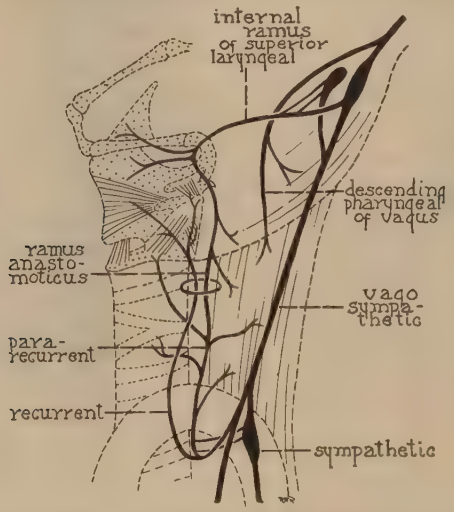


Fig. 2 Pattern of laryngeal nerves in dog. Diagram based on serial sections and binocular dissections.

recurrent below. Stimulation and degeneration experiments are necessary to unravel this anastomosis.

Although Nordland ('30) could find a ramus anastomoticus in only three out of nineteen human cadavers, it was present in ten out of twelve of my dissections. It is not nearly as large in man as in dogs and often forms a very slender ramus which might possibly be missed in the dissection of human cadavers. In the two cases in which the ramus anastomoticus was absent the terminal twig of the internal ramus of the superior laryngeal nerve simply ran directly to the pha-

ryngeal and esophageal mucosa without a preliminary union with the recurrent nerve. This suggests that the ramus anastomoticus is not nearly as important in man as in dogs and also that it probably innervates only the pharyngeal and esophageal mucosa overlying the back part of the larynx.

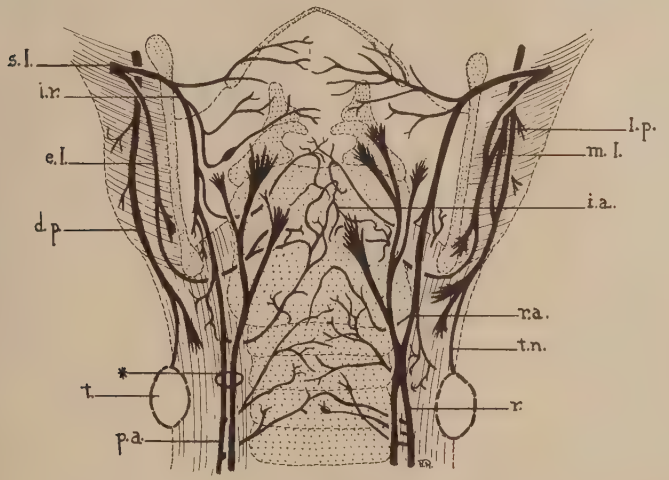


Fig. 3 Laryngeal nerves in the dog as viewed from behind (posterior aspect). Graphic reconstruction from a complete series of sections through a pup's larynx stained with pyridine silver. *dp*, descending pharyngeal of vagus; *el*, external branch of superior laryngeal nerve; *ia*, internal anastomosis between recurrent and superior laryngeal nerves; *ir*, internal ramus of superior laryngeal nerve; *lp*, laryngeal plexus; *ml*, median laryngeal nerve; *pa*, pararecurrent; *r*, recurrent; *ra*, ramus anastomoticus; *sl*, superior laryngeal nerve; *t*, thyroid gland; *tn*, thyroid nerve; *, lying in same sheath, but separable and not fused.

In addition to this external anastomosis, there is an internal anastomosis between the recurrent and superior laryngeal nerves (fig. 3, *ia*). This is simply a plexus formation in the mucosal wall of the larynx and has no more significance than the nerve plexuses in the mucosa of other parts of the respiratory tube.

STIMULATION EXPERIMENTS IN DOGS

The recurrent proper was found to innervate the laryngeal muscles only and therefore was of no significance to the problem. Stimulation of the caudal stump of the sectioned internal ramus of the superior laryngeal nerve (fig. 4, *a*) produced mucous secretion on the same side in the larynx and

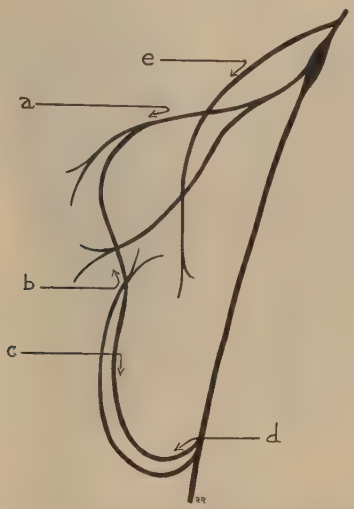


Fig. 4 Diagram showing levels of stimulation experiments in dogs. *a*, caudal stump of sectioned internal ramus of superior laryngeal nerve; *b*, cephalic stump of sectioned ramus anastomoticus or pararecurrent in its upper third; *c*, caudal stump of pararecurrent sectioned in its middle or lower third; *d*, cephalic stump of pararecurrent sectioned low in the neck close to its origin; *e*, caudal stump of sectioned descending pharyngeal of vagus.

overlying pharynx, and in the trachea and esophagus to the lower fourth of the neck. This was evidenced by a hastened formation of mucous drops on the mucous-membrane surfaces during stimulation. These impulses were found to travel by way of the ramus anastomoticus and pararecurrent nerves. Reflex secretion due to sensory stimulation through the pararecurrent was precluded by sectioning the latter at its origin from the recurrent or vagus.

Stimulation of the cephalic stump of the sectioned ramus anastomoticus or pararecurrent in its upper third (fig. 4, *b*) gave swallowing reflexes and signs of pain. These impulses were found to come from the pharyngeal mucosa overlying the larynx and from the mucosa of the upper third of the esophagus. They passed centrally through the superior laryngeal nerve and the vagus to the medulla.

Stimulation of the caudal stump of the pararecurrent sectioned in the middle or lower third of the neck (fig. 4, *c*) gave increased mucous secretion, as described above. Coughing reflexes were elicited from the trachea and posterior infracordal mucosa of the larynx. Swallowing reflexes were elicited from the esophageal mucosa up to the upper third in the neck. These swallowing and coughing reflexes were rechecked by direct stimulation of the tracheal and esophageal mucosa with an applicator. They depended on the intactness of the pararecurrent below this level and of the vagus for their manifestation.

Stimulation of the cephalic stump of the pararecurrent sectioned low in the neck close to its origin (fig. 4, *d*) produced mucous secretion from the trachea and esophagus in the lower fourth of the neck. There was also a contraction of the esophageal musculature in the lower two-thirds of the neck. The superior laryngeal nerve was sectioned in this group of experiments to prevent any chance of reflex action centrally. The musculature of the upper third of the esophagus was found to be innervated by a branch of the pharyngeal ramus of the vagus (fig. 4, *e*).

## NORMAL HISTOLOGY OF THE RAMUS ANASTOMOTICUS AND PARARECURRENT NERVES

The ramus anastomoticus and its branches to the pharyngeal and esophageal mucosa contain large, medium, and small myelinated and black and brown unmyelinated fibers (figs. 6 and 7). The small myelinated fibers (fig. 6, *a*) are the most numerous and are in all probability preganglionic parasympathetic fibers, as will be discussed under the de-

generation experiments. The large and medium-sized myelinated fibers (fig. 6, *b*) possibly carry tactile sensation from the mucosa. The small black unmyelinated fibers (fig. 7, *c*) are arranged in groups and correspond to the pain fibers described by Ranson ('18). They are relatively quite scarce as are the brown unmyelinated fibers which are undoubtedly postganglionic sympathetic in nature (fig. 7, *d*).

The pararecurrent is composed of the same type of fibers except for slight differences in the lower half of the neck. There are added some large and finely myelinated fibers which are distributed to the esophageal musculature. These are probably motor and muscle sense, respectively, for they correspond to the same type of fibers innervating the laryngeal muscles.[2] That painful sensations may arise in the upper part of the trachea at least is indicated by the presence of small black pain-type fibers in the pararecurrent.

Variable groups of ganglion cells were found along the course of the internal ramus of the superior laryngeal nerve and its branches, including the ramus anastomoticus. These cells were multipolar (fig. 9, *a*) and looked like peripheral parasympathetic cells found elsewhere in the body. They were included in fascicles (fig. 10) containing small myelinated preganglionic (*a*) and unmyelinated postganglionic fibers (*b*). Where some of the branches of the ramus anastomoticus passed through the posterior crico-arytenoid muscle, cells could be seen intramuscularly. Where there was a plexus formation with the recurrent nerve, cells could also be seen on some of its branches.

## DEGENERATION EXPERIMENTS

Sectioning of the superior laryngeal nerve caused complete degeneration of the ramus anastomoticus (figs. 11 and 12), except for a few black unmyelinated fibers (fig. 12, *a*). These intact fibers came from the ganglion cells present in the nerve distal to the point of sectioning. There were no

[2] Innervation of the larynx. I. Innervation of laryngeal muscles. Am. J. Anat., v. 51, no. 2, Nov. 15.

processes running centrally from these cells to the level of sectioning which tends to disprove the suggestion that these cells might be similar to the sensory cells in the spinal ganglion.

The almost complete degeneration of the ramus anastomoticus extended on down into the pararecurrent as far as its uppermost branch. From here down fibers began filling in (figs. 13 and 14) until in its middle third the pararecurrent was apparently intact. All twigs to the mucosa of the upper third of the esophagus in the neck, whether of ramus anastomoticus or pararecurrent origin, were completely degenerated except for a few postganglionic parasympathetic fibers. This agrees with the findings obtained from stimulation experiments.

Sectioning of the vagus or of the recurrent and pararecurrent nerves low in the neck gave degeneration of all the ascending fibers. This was evidenced by complete degeneration of the pararecurrent and its branches in the lower fourth of the neck. Above this the pararecurrent contained small myelinated fibers (fig. 15, *a*) in increasing numbers as the nerve was followed cephalad. Aside from these fibers, the pararecurrent and its branches were completely degenerated except for that portion supplying the mucosa of the esophagus in its upper third. The ramus anastomoticus was intact in all these experiments where the ascending fibers were wiped out by resection of the pararecurrent or vagus below the origin of the superior laryngeal nerve (figs. 16 and 17).

The small myelinated fibers coursing in the pararecurrent from above to its lower fourth in the neck were interpreted as preganglionic parasympathetic fibers. They apparently carry the secretory impulses to the trachea and esophagus which were demonstrated in the stimulation experiments. Their appearance is also that of parasympathetic fibers which have been described in other parts of the nervous system.

## SUMMARY AND DISCUSSION

The attempt has been made to unravel the anastomosis between the superior and recurrent laryngeal nerves by means of anatomical methods and stimulation and degeneration ex-

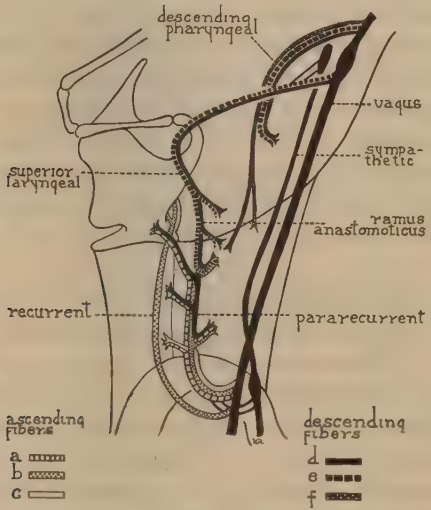


Fig. 5 Diagrammatic summary of components contained in the ramus anastomoticus, recurrent, and pararecurrent laryngeal nerves, and the descending twig of the pharyngeal ramus of the vagus. Ascending fibers: *a*, large and medium-sized myelinated sensory and black pain-type and brown unmyelinated postganglionic sympathetic fibers to mucous membrane of the trachea, lower larynx, and esophagus; *b*, large myelinated motor and finely myelinated sensory fibers to laryngeal and esophageal musculature; *c*, small myelinated preganglionic parasympathetic secretory fibers to the tracheal and esophageal mucous membrane. Descending fibers: *d*, small myelinated preganglionic parasympathetic secretory fibers to larynx, trachea, and esophagus; *e*, large and medium-sized myelinated sensory and black pain-type and brown unmyelinated postganglionic sympathetic fibers to mucous membrane of esophagus; *f*, large myelinated motor and finely myelinated sensory fibers to esophageal musculature.

periments. Each of these separately give information which is inconclusive, but when combined serve to supplement and confirm one another. The composite picture of the findings is best illustrated by an analytical diagram (fig. 5).

The following are the ascending fibers as determined by stimulation and degeneration experiments. One component (*a*) supplies the mucosa of the trachea, the posterior infracordal region of the larynx, and the esophagus in the lower two-thirds of the neck. This component contains mostly large and medium-sized myelinated fibers probably carrying sensation from the mucosal surfaces. There are also a few unmyelinated postganglionic sympathetic fibers in this component and a few groups of small black unmyelinated pain-type fibers. Another group of fibers (*b*) supplies the esophageal musculature in the lower two-thirds of the neck. These fibers are large and finely myelinated and are similar to those supplying the laryngeal muscles. A third functional component (*c*) is formed by the small myelinated preganglionic parasympathetic fibers which carry secretory impulses to the trachea and esophagus in the lower fourth of the neck.

The descending fibers from the internal ramus of the superior laryngeal nerve are as follows: Small myelinated preganglionic parasympathetic fibers (*d*) are in the majority. They carry secretory impulses to the tracheal and esophageal mucosa of the upper three-fourths of the neck as demonstrated by stimulation experiments. The mucosa of the esophagus in its upper third in the neck is innervated by large and medium-sized myelinated fibers, probably tactile in nature (*e*). Included in this component are a few unmyelinated pain and postganglionic sympathetic and parasympathetic fibers. The esophageal musculature in its upper third in the neck is supplied by large and finely myelinated fibers derived from the pharyngeal ramus of the vagus (*f*).

The ganglion cells along the superior laryngeal nerve are undoubtedly peripheral parasympathetic ganglion cells for the following reasons. First, they are intimately associated with fascicles containing both pre- and postganglionic parasympathetic fibers. Secondly, they are multipolar and have the appearance of parasympathetic cells found elsewhere in the body. And,thirdly, they have no central processes which preclude them from being classed as sensory in type. These

cells probably correspond to peripheral parasympathetic cells found elsewhere, as in the nerves to the heart or the intestines.

These ganglion cells in the superior laryngeal nerve have been described by a number of investigators. Elze ('03) found them along the ramus anastomoticus and its branches in man. He considered these to be the same as the unipolar type of sensory cell found in the spinal ganglia. Geronzi ('04) found ganglion cells in the posterior crico-arytenoid and cricothyroid muscles of the larynx. He thought that in some cases processes of these cells terminated on motor end plates and concluded that the cells were secondary reflex centers for respiration analogous to sympathetic cells in the heart. The true explanation, however, would seem to be that these are merely parasympathetic cells along the nerves piercing these muscles to supply the underlying mucosa. More recently, Nonidez ('31) has described ganglion cells in the superior laryngeal in relation to the innervation of the thyroid gland. He tends also to the conclusion that these cells are peripheral parasympathetic in nature.

It is interesting to speculate on the variations of the ramus anastomoticus, the pararecurrent, and the recurrent in the different species. Rethi ('98), Katzenstein ('00), and Schulz and Dorendorf ('04) all found that in dogs the afferent impulses from the upper third of the recurrent nerve[3] in the neck were wiped out by section of the ramus anastomoticus or superior laryngeal. These reflexes were pain and swallowing. They agree uniformly that in cats and rabbits, on the other hand, the reaction of the recurrent nerve is in no way affected by section of the ramus anastomoticus or superior laryngeal nerve. Kokin ('97) found that the superior laryngeal nerve supplied secretory fibers to the larynx and upper half of the trachea through the ramus anastomoticus and pararecurrent nerves in dogs. In cats the secretory fibers for the trachea and lower larynx were derived entirely through the recurrent from below. In the dog the ramus anastomoticus is much larger than in other species, including

[3] Probably that part of the recurrent termed the pararecurrent in this paper.

man, approaching in size the recurrent. Schulz and Dorendorf ('04) report the ape as reacting in the same way as cats and rabbits, while goats react like dogs in the effect that section of the ramus anastomoticus has on the reactions of the recurrent and pararecurrent nerves. The above data strongly suggest that the ramus anastomoticus in man, cats, and rabbits is relatively unimportant and probably innervates only the uppermost portion of the esophageal mucous membrane and the pharyngeal mucosa lying over the posterior surface of the larynx.

The complexity of the above arrangement is best explained by the migration of structures in this region during embryonic development (Frazer, '10).

## CONCLUSIONS

1. The ramus anastomoticus between the superior and recurrent laryngeal nerves exhibits a wide variety of patterns ranging from a single trunk to a plexus formation.

2. The ramus anastomoticus contains only fibers from the superior laryngeal nerve. These are distributed for the most part through branches of the pararecurrent nerve in dogs or the recurrent nerve in man.

3. In man these fibers probably innervate only the lower pharyngeal and upper esophageal mucosa. In dogs the ramus anastomoticus supplies in addition secretory fibers to the mucosa of the infracordal part of the larynx and the trachea and esophagus as far as the lower fourth of the neck. These impulses pass by way of the pararecurrent nerve.

4. The fibers of the ramus anastomoticus innervating the mucous membrane consist of large, medium, and small myelinated fibers and a few unmyelinated fibers. The large and medium-sized fibers are possibly tactile in nature. The few groups of black unmyelinated fibers probably conduct pain impulses from the mucosa. There are also a few larger brown unmyelinated fibers which look like postganglionic sympathetic fibers probably carrying vasomotor impulses.

5. The small myelinated fibers are probably preganglionic parasympathetic fibers. They carry the secretory impulses as demonstrated by stimulation and degeneration experiments.

6. The ganglion cells found along the superior laryngeal nerve and its ramus anastomoticus were multipolar and resembled peripheral parasympathetic cells in their relationships and morphology.

7. Cells found in the laryngeal muscles are undoubtedly parasympathetic cells occurring on nerve itwigs piercing these muscles to supply the underlying mucous membrane.

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# PLATES

## PLATE 1

### EXPLANATION OF FIGURES

6 Ramus anastomoticus, osmic preparation. *a*, small myelinated preganglionic parasympathetic fibers; *b*, large and medium-sized sensory fibers. Photo. × 127.

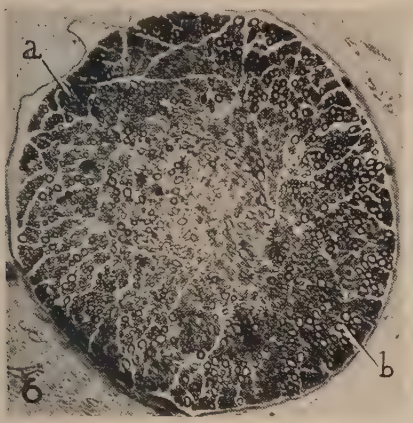
7 Ramus anastomoticus, pyridine-silver preparation. *c*, groups of small black unmyelinated pain type of fibers; *d*, brown unmyelinated postganglionic sympathetic fibers. Photo. × 127.

8 Pararecurrent, pyridine-silver stain. *a*, groups of small black unmyelinated pain type of fibers. Photo. × 300.

9 Peripheral parasympathetic ganglion cells along the internal ramus of the superior laryngeal nerve. Pyridine-silver stain. *a*, cell showing multipolar processes. Photo. × 300.

10 Fascicle of internal ramus of superior laryngeal nerve showing parasympathetic ganglion cells and *a*) small myelinated preganglionic fibers and, *b*) unmyelinated postganglionic fibers. Osmic preparation. Photo. × 750.

11 Ramus anastomoticus: *a*) degenerated and recurrent, *b*) intact after resecting superior laryngeal nerve. Osmic stain. Photo. × 70.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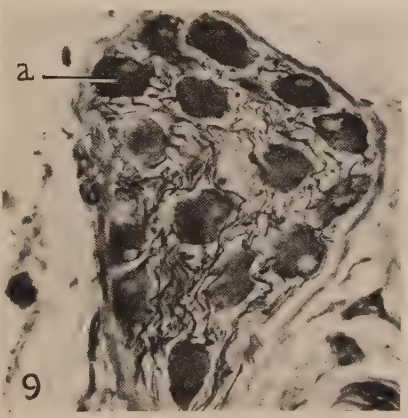
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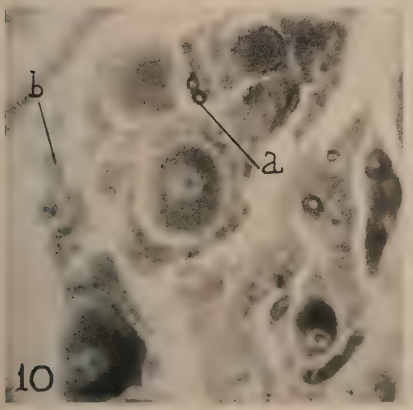
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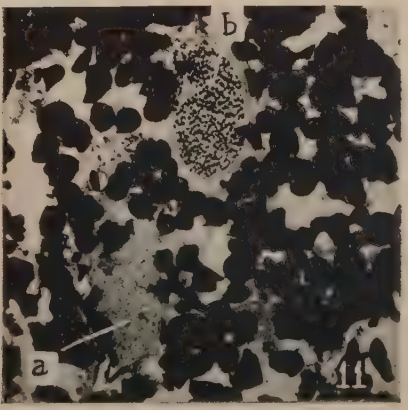
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## PLATE 2

EXPLANATION OF FIGURES

12 Ramus anastomoticus degenerated, except for black unmyelinated postganglionic parasympathetic fibers (*a*) from ganglion cells in internal ramus of superior laryngeal nerve above. Recurrent (*b*) intact. Superior laryngeal nerve resected thirty days previously. Pyridine-silver preparation. Photo. × 170.

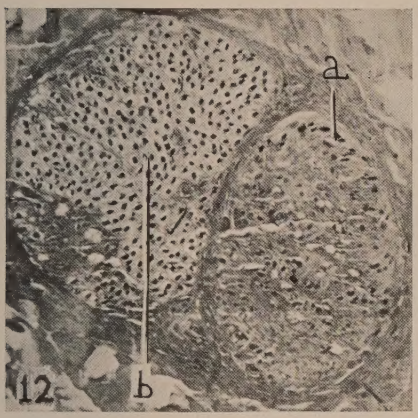
13 Partially degenerated pararecurrent after resecting superior laryngeal nerve. Osmic stain. Photo. × 170.

14 Partially degenerated pararecurrent after resecting superior laryngeal nerve. Pyridine-silver stain. Photo. × 127.

15 Recurrent and pararecurrent degenerated after resecting low in neck close to origin. *a*, intact small myelinated preganglionic parasympathetic fibers coursing in pararecurrent from ramus anastomoticus above. Osmic preparation. Photo. × 70.

16 Ramus anastomoticus: *a*) intact and recurrent, *b*) degenerated after resecting vagus or pararecurrent and recurrent low in neck. Osmic stain. Photo. × 70.

17 Ramus anastomoticus: *a*) intact and recurrent, *b*) degenerated after resecting vagus or pararecurrent and recurrent low in neck. Pyridine-silver stain. Photo. × 70.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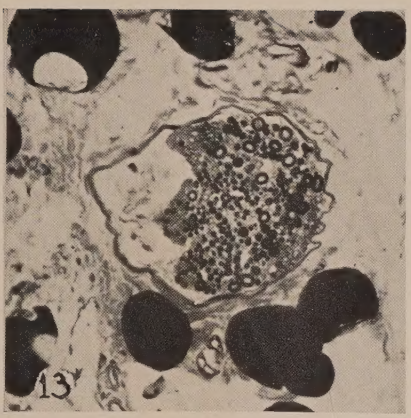
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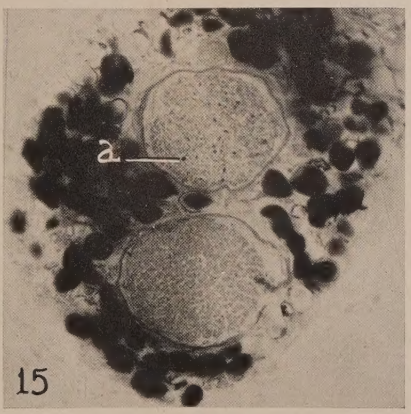
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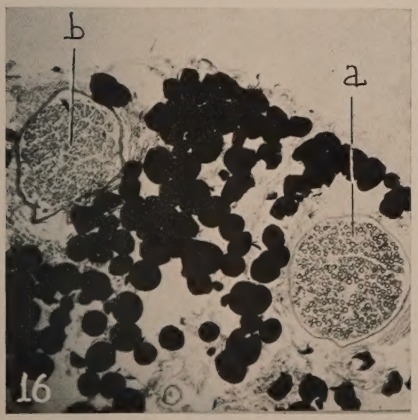
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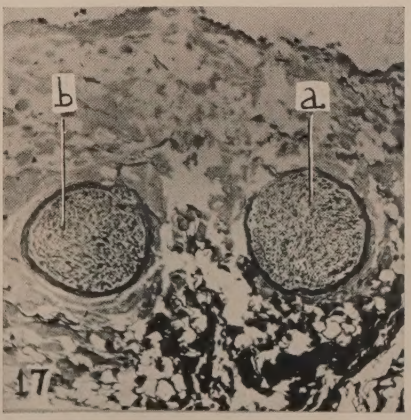
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